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Attorney Docket No. 63637-0102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

Reggie Y.C. LO, Anthony B. SCHRYVERS and Andrew A. POTTER

Serial No.: (New Application)

Filed: November 29, 1996

Title: TRANSFERRIN BINDING PROTEINS OF PASTEURILLA
HAEMOLYTICA AND VACCINES CONTAINING SAMEAssistant Commissioner for Patents
United States Patent and Trademark Office
Washington, D.C. 20231Sir: NEW APPLICATION TRANSMITTAL

Attached is an unexecuted application for filing in the United States Patent and Trademark Office, including eighty-four (84) pages of specification, twenty-nine (29) claims, seven (7) of which are independent, an Abstract and thirty-four (34) sheets of drawings, Figures 1-29.

This application is being filed without the filing fee and with an unexecuted Declaration under 37 C.F.R. § 1.53(d). Accordingly, applicants are believed to be entitled to a filing date based upon the U.S. Patent and Trademark Office's receipt of the attached specification and drawings. Therefore, it is respectfully requested that a filing date of November 29, 1996 be granted this application. The filing fee and executed Declaration will be timely filed.

The undersigned's Deposit Account No. is 02-0375. A duplicate of this sheet is attached.

Respectfully submitted,
BAKER & BOTTS, L.L.P.

Date: November 29, 1996

By: 

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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United States

Title: Transferrin Binding Proteins of *Pasteurella haemolytica*
and Vaccines Containing Same

Inventor(s): Reggie Y.C. Lo, Anthony B. Schryvers
and Andrew A. Potter

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Title: Transferrin Binding Proteins of *Pasteurella haemolytica* and Vaccines
Containing Same 11/29/96

FIELD OF THE INVENTION

5 The invention relates to novel transferrin binding proteins of *Pasteurella haemolytica*, truncations, analogs, homologs and isoforms thereof; nucleic acid molecules encoding the proteins and truncations, analogs, and homologs of the proteins; vaccines containing the proteins; antibodies against the proteins; and, uses of the proteins and nucleic acid molecules.

BACKGROUND OF THE INVENTION

10 Members of the genus *Pasteurella* comprise a group of related bacterial species that are important pathogens of ruminants. This group includes the species *Pasteurella haemolytica* which has been classified into two biotypes, A and T, on the basis of sugar utilization, and into 16 serotypes which are recognized on the basis of their somatic antigens (Biberstein, E. L. et al., 15 1960; Fraser et al., 1982). The T-type strains of *P. haemolytica*, characterized by utilization of trehalose, have been recently reclassified as a new species *P. trehalosi* (Sneath, P.H.A. et al., 1990).

16 Pneumonic pasteurellosis caused by *Pasteurella haemolytica* is a major economic problem to the cattle, sheep and goat industries world-wide. Shipping fever, a variation of this 20 disease, is a major problem in the cattle industry in North America and is almost exclusively caused by type A1 strains of this species (Babiuk, L.A. and S. D. Acres, 1984). Serotype A2 is the most prevalent disease-causing type in sheep but other serotypes may be important in sheep and goats (Gilmour and Gilmour, 1991). The related species, *Pasteurella trehalosi* (formerly know as T-type *P. haemolytica*) is the causative agent of septicemia in lambs, a problem plaguing the 25 sheep industry particularly in the United Kingdom. Similarly, strains of the related species *Pasteurella multocida*, are responsible for haemorrhagic septicemia, a serious infection in cattle and water buffalo, which is particularly serious in South East Asia.

30 Vaccination is a desired method of control for pasteurellosis in ruminants but success has been limited by the lack of immunizing preparations that induce protection against all disease-causing serotypes, particularly if a vaccine effective for all ruminants is considered. Killed whole cell vaccines elicited inconsistent levels of protection and antibody response in calves (Wilkie, B.N., 1980). Homologous vaccines containing sodium salicylate extracts (SSEs) protected sheep against diseases due to serotypes A1, A6 and A9 (Gilmour et al., 1983) but not against the more epidemic serotype A2 (Fraser et al., 1982). An exotoxin produced by *P.* 35 *haemolytica* which is specifically lethal to leucocytes and alveolar macrophages from ruminants (Benson et al., 1978) has shown a lot of promise as a vaccine candidate in protection experiments in calves and sheep (13,35) but there is limited protection against heterologous

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serotypes (33). The inclusion of proteins induced under iron-limited growth conditions into a vaccine for pasteurellosis in lambs has been implicated in enhanced protection (15).

Previous studies have established that the ability of pathogenic bacteria to acquire iron *in vivo* is a critical factor in their pathobiology (7,11). One mechanism of iron retrieval from the host iron-binding glycoprotein, transferrin, involves direct binding of transferrin by surface receptors on the bacteria and the removal of iron from transferrin and uptake into the cell (21). Schryvers (1992) describes the isolation of transferrin receptor proteins from various bacterial pathogens using affinity chromatography. The transferrin receptor has been shown to consist of two proteins, called transferrin binding protein 1 or A (Tbp1 or TbpA) and transferrin binding protein 2 or B (Tbp2 or TbpB). The receptor-mediated type of iron uptake has been demonstrated to operate in serotype A bovine strains of *P. haemolytica* (26). Cells of *P. haemolytica* growing *in vitro* under iron-limited conditions express a number of iron-repressible outer membrane proteins (IROMPs) identical to those produced by cells recovered *in vivo* from infected sites in animals with pasteurellosis (9,10). Especially prominent among these proteins were those of molecular sizes 100, 77, 70 and 60 Kda (9,10). The 100 Kda protein has been identified as one of the host specific transferrin receptors in bovine isolates (26) while some of the other IROMPs had been suggested as possibly associated with the 100 Kda protein in an iron acquisition receptor complex (26). The role of the IROMPs expressed by *P. haemolytica* from lambs (10) in iron acquisition has not been elucidated, neither is it known if similar proteins are expressed by goat isolates.

P. haemolytica acquires iron from bovine host transferrin by a receptor-mediated type of mechanism. The proposal that bacteria with this type of iron acquisition mechanism may be solely dependent upon their surface receptor for iron acquisition *in vivo* (29) implies that they can only cause disease in those hosts whose transferrin is recognized by their surface receptors. *P. haemolytica* has been reported to cause disease in cattle, sheep and goats and accordingly their surface receptors would be expected to recognize these hosts' transferrins. Therefore it is important to determine whether sheep and goat isolates also possessed transferrin receptors involved in iron acquisition, to evaluate their specificities for different ruminant transferrins and to determine if there is antigenic relatedness amongst the surface receptors from the different strains causing pneumonic pasteurellosis in cattle, sheep and goats.

SUMMARY OF THE INVENTION

Transferrin receptors were identified in a collection of *Pasteurella haemolytica* (and *P. trehalosi*) strains of various serotypes and biotypes (A and T) from cattle, sheep and goats. Growth studies, binding studies and affinity isolation experiments demonstrated that these receptors had identical specificities which recognized transferrins from cattle, sheep and goats. This indicates that there are conserved regions on the receptor proteins, involved in ligand binding, which are accessible at the cell surface.

Antisera prepared against the individual purified receptor proteins (TbpA and TbpB) from a serotype A1 strain of *P. haemolytica* demonstrated considerable crossreactivity against receptor proteins from a representative selection of strains. The cross-reactivity was also observed against intact cells indicating that there are conserved immunological epitopes at the cell surface which could serve as targets for the host's immune effector mechanisms.

The present inventors have cloned, sequenced and expressed *tbpA* and *tbpB* genes encoding the transferrin receptor proteins, TbpA and TbpB (also referred to herein as Tbp1 and Tbp2, respectively), from *Pasteurella haemolytica* A1. The genes were organized in an operon arrangement of *tbpB-tbpA*. The *tbpB* gene was preceded by putative promoter and regulatory sequences, and followed by a 96 base pair intergenic sequence in which no promoter regions were found, suggesting that the two genes are coordinately transcribed. The deduced amino acid sequences of the TbpA and TbpB proteins had regions of homology with the corresponding *Neisseria meningitidis*, *N. gonorrhoeae*, *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae* Lbp and Tbp proteins. The intact *tbpB* gene was expressed in a T7 expression system and the resulting recombinant TbpB protein retained the functional bovine transferrin binding characteristics. The availability of the recombinant TbpB enabled the inventors to demonstrate its specificity for ruminant transferrin, its ability to bind both the C-and N-terminal lobes of bovine transferrin, and its preference for the iron-loaded form of this protein.

The present inventors also significantly found that vaccination with a formulation containing *P. haemolytica* TbpA and TbpB provided significant protection against experimental bovine pneumonic pasteurellosis. Immunization with two doses of TbpB also provides protection.

Broadly stated, the present invention provides a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpA protein, or a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpB protein. The TbpA and TbpB proteins bind ruminant transferrins and function in receptor-mediated iron acquisition by *P. haemolytica* in its ruminant hosts. The TbpA protein is approximately 100kDa, and TbpB is approximately 60 kDa in size.

In an embodiment of the invention, the purified and isolated nucleic acid molecules comprise a sequence encoding a TbpA protein having the amino acid sequence as shown in Figure 22 or SEQ. ID. NO:2, or a sequence encoding a TbpB protein having the amino acid sequence as shown in Figure 24 or SEQ. ID. NO:4. In a preferred embodiment of the invention, the purified and isolated nucleic acid molecules comprise a sequence encoding a TbpA protein and having the nucleic acid sequence as shown in Figure 21 or SEQ. ID. NO:1, or a sequence encoding a TbpB protein having the nucleic acid sequence as shown in Figure 23 or SEQ. ID. NO:3.

The invention also contemplates (a) nucleic acid molecules comprising a sequence encoding a truncation of TbpA or TbpB which is unique to the protein, an analog or homolog of TbpA or TbpB or a truncation thereof, (herein collectively referred to as "TbpA related proteins" or "TbpB related proteins", respectively); (b) a nucleic acid molecule comprising a sequence

which hybridizes under high stringency conditions to the full length nucleic acid encoding TbpA or TbpB, having the amino acid sequences as shown in Figures 22 and 24 respectively, or to a TbpA or TbpB related protein; (c) a nucleic acid molecule comprising a sequence which hybridizes under high stringency conditions to the full length nucleic acid sequence of the *tbpA* or *tbpB* genes having the sequences as shown in Figures 21 or SEQ. ID. NO:1, or Figure 23 or SEQ. ID. NO:3, respectively.

The invention further contemplates a purified and isolated double stranded nucleic acid molecule containing a nucleic acid molecule of the invention, hydrogen bonded to a complementary nucleic acid base sequence.

10 The nucleic acid molecules of the invention may be inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Accordingly, recombinant expression vectors adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements operatively linked to the
15 nucleic acid molecule.

The recombinant expression vector can be used to prepare transformed host cells expressing TbpA and/or TbpB, or a TbpA or a TbpB related protein. Therefore, the invention further provides host cells containing a recombinant molecule of the invention.

The invention further provides a method for preparing a novel TbpA or TbpB, and
20 TbpA or TbpB related proteins, utilizing the purified and isolated nucleic acid molecules of the invention. In an embodiment a method for preparing TbpA or TbpB is provided comprising (a) transferring a recombinant expression vector of the invention into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of TbpA or TbpB; and (d) isolating the recombinant
25 TbpA or TbpB.

The invention further broadly contemplates a purified and isolated TbpA or TbpB which binds to ruminant transferrin, preferably obtained by culturing a host cell containing a recombinant expression vector of the invention. In an embodiment of the invention, a purified TbpA or TbpB is provided which has the amino acid sequence as shown in Figure 22 or Figure 24
30 respectively. The invention also includes truncations of the protein and analogs, homologs, and isoforms of the protein and truncations thereof (i.e., "TbpA or TbpB related proteins").

The TbpA and TbpB, or TbpA and TbpB related proteins of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins.

35 The invention further contemplates antibodies having specificity against an epitope of TbpA or TbpB, or TbpA or TbpB related proteins of the invention. Antibodies may be labelled with a detectable substance and they may be used to detect the TbpA or TbpB, or TbpA or TbpB related proteins of the invention in samples.

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The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to TbpA or TbpB, or TbpA or TbpB related proteins of the invention. Thus, the invention also relates to a probe comprising a sequence encoding TbpA or TbpB, or TbpA or TbpB related proteins. The probe may be labelled, 5 for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays one or more of the properties of TbpA or TbpB.

The invention still further provides a method for identifying a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, comprising reacting TbpA or TbpB, or TbpA or TbpB related proteins, or an activated 10 form thereof, with at least one substance which potentially can bind with TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, and assaying for complexes, for free substance, for 15 non-complexed TbpA or TbpB or a TbpA or TbpB related proteins, or an activated form thereof. Substances which potentially can bind TbpA or TbpB, or TbpA or TbpB related proteins, include transferrins, particularly ruminant transferrins, analogs and derivatives of transferrins and antibodies against TbpA and TbpB, or TbpA or TbpB related proteins.

Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of TbpA or TbpB, or TbpA or TbpB related 20 proteins, and a substance which binds to TbpA or TbpB, or TbpA or TbpB related proteins or an activated form thereof. In an embodiment, the method comprises providing a known concentration of TbpA or TbpB, or TbpA or TbpB related proteins, with a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins and a suspected agonist or 25 antagonist substance under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB, or TbpA or TbpB related proteins. In a preferred embodiment of the invention, the substance is a ruminant transferrin, analog, derivative or part thereof or an antibody against TbpA or TbpB, or TbpA or TbpB related 30 proteins.

Substances which affect expression of TbpA or TbpB, or TbpA or TbpB related proteins, may also be identified using the methods of the invention by comparing the pattern and level of expression of TbpA or TbpB, or TbpA or TbpB related proteins of the invention, in cells in the presence, and in the absence of the substance.

35 The substances identified using the methods of the invention may be used in the treatment of animals, particularly ruminants infected with *P. haemolytica* and accordingly they may be formulated into pharmaceutical compositions for administration to ruminants, such

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as cattle, sheep and goats suffering from infection with *P. haemolytica* or exposed to infection by *P. haemolytica*.

The present inventors have demonstrated that the TbpA or TbpB, or TbpA or TbpB related proteins of the invention, are immunogenic. Therefore, the invention also relates to antibodies against the TbpA or TbpB, or TbpA or TbpB related proteins of the invention. In an embodiment, the antibodies are cross reactive against TbpA or TbpB or TbpA, or TbpB related proteins, from a wide range of serotypes of *P. haemolytica*. The antibodies may be used in the diagnosis and treatment of *P. haemolytica* infection and may be used, for example, in passive immunization to treat or prevent diseases in ruminants caused by *P. haemolytica*.

The invention further includes vaccine compositions comprising the TbpA or TbpB, or TbpA or TbpB related proteins of the invention, either alone, or in combination. The invention still further includes methods of immunizing a host, preferably a ruminant host against infection by *P. haemolytica* by administering therapeutically effective amounts of such vaccines. The present inventors have demonstrated that different strains of *P. haemolytica*, from a range of ruminants, are able to bind and utilize a range of ruminant transferrins. Thus it is contemplated that the vaccine compositions of the invention will be useful as broad spectrum vaccines suitable for immunizing a range of ruminants, such as sheep, cows and goats against infection with a wide range of *P. haemolytica* biotypes and serotypes.

The invention also contemplates the use of nucleic acid molecules of the invention encoding TbpA or TbpB, or TbpA or TbpB related proteins, in a recombinant viral vector vaccine for augmenting the immune response of a ruminant to *P. haemolytica* or for treating *P. haemolytica* infection. Recombinant viral vectors may be constructed using techniques known in the art.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a schematic diagram of the PCR procedure (a), and the 0.8 kb PCR product amplified by Tbp1 primer and primer left (b);

Figure 2 is a restriction endonuclease map of *tbp* plasmids 9, 10, and 482;

Figure 3 is a preliminary nucleotide sequence of *P. haemolytica* *tbpA* and *tpbB*;

Figure 4 is the promoter region of *P. haemolytica* *tbpB* (PHTBPB);

Figure 5 is a blot of Southern hybridization of *P. haemolytica* genomic DNA digested with *ClaI* and probed with *tbpA* gene;

Figure 6 is a blot of Southern hybridization of *P. haemolytica* genomic DNA digested with *Hind* III and *Bam*HI and probed with the *tpbA* gene;

Figure 7 is a blot of Southern hybridization of *A. suis* 37114, *A. pleuropneumoniae* CM5 and Shope 4074 genomic DNA digested with various restriction endonucleases and probed with *P. haemolytica tpbA*;

Figure 8 are restriction maps of the *tpbA*, *tpbB* regions in *P. haemolytica* A1, *A. pleuropneumoniae* CM5, Shope 4074, and *A. suis* 3714;

Figure 9 shows an alignment of the amino acid of Tbp1 of *P. haemolytica* A1,(PHTBP) and the Tbp1 of *N.gonorrhoeae* (NGTBP1) and *N. meningitidis* (NM1);

Figure 10 shows an alignment of the amino acid of Tbp1 of *P. haemolytica* A1,(PHTBP) and the *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7);

Figure 11 is a dendrogram illustrating the genetic relatedness among *P. haemolytica* A1,(PHTBP) and the Tbp1 of *N.gonorrhoeae* (NGTBP1), *N. meningitidis* (NM1) and *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7);

Figure 12 is a peptide alignment between *P. haemolytica* A1 Tbp1 and TonB-dependent outer membrane receptors of *E. coli*;

Figure 13 is a blot showing T7 analysis of the *P.haemolytica* Tbp1 protein;

Figure 14 is a Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E.coli* HB101;

Figure 15 is a Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E.coli* HB101 using sera raised in calves to soluble antigens by vaccination with Presponse®;

Figure 16 is a blot showing the binding of labelled transferrins by iron-deficient bacterial membranes;

Figure 17 is an immunoblot showing isolation of receptor proteins with transferrin affinity columns;

Figure 18 are immunoblots showing immunological analysis of receptor proteins from different serotypes of *P. haemolytica* from bovine, sheep, and goats, where Panel A is with anti-TbpB serum and Panel B is with anti-TbpA serum;

Figure 19 are blots showing the binding of labelled transferrin and anti-receptor antibody by intact cells;

Figure 20 is a map of the *P. haemolytica tpb* operon (Top) and *P. haemolytica tpb* operon (Top) and regulatory sequences (Bottom); *tpbA* and *tpbB* are the genes encoding for TbpA and TbpB, respectively; p, is the putative promoter region preceding *tpbB* and denoted as -35 and -10 sites at the bottom;

Figure 21 and SEQ ID NO:1 show the DNA sequence of the *tpbA* gene from *P. haemolytica* strain h196;

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Figure 22 and SEQ ID NO:2 show the predicted amino acid sequence of the TbpA protein from *P. haemolytica* strain h196;

Figure 23 and SEQ ID NO:3 show the DNA sequence of the *tbpB* gene from *P. haemolytica* strain h196;

5 Figure 24 and SEQ ID NO:4 show the predicted amino acid sequence of the TbpB protein from *P. haemolytica* strain h196;

Figure 25 is a blot showing the results of a solid-phase HRP-Tf binding assay;

Figure 26 are blots showing silver stain (Panel A), and western blot (Panel B) studies with anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1;

10 Figure 27 is a blot showing the results of cross-reactivity studies with monospecific anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1 against intact cells;

Figure 28 shows a gel with restriction endonuclease digestion patterns of PCR-amplified *tbpA* (Panel A) and *tbpB* (Panel B) genes from *P. haemolytica* and *P. trehalosi* strains; and

15 Figure 29 is a gel showing PCR amplification of variable segments of the *tbpA* (Panel A) and *tbpB* (Panel B) genes.

DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations for the amino acid residues are used throughout the specification: A, Ala - alanine; C, Cys - cysteine; D, Asp- aspartic acid; E, Glu - glutamic acid; F, Phe - phenylalanine; G, Gly - glycine; H, His - histidine; I, Ile - isoleucine; K, Lys - lysine; L, Leu - leucine; M, Met - methionine; N, Asn - asparagine; P, Pro - proline; Q, Gln - glutamine; R, Arg - arginine; S, Ser - serine; T, Thr - threonine; V, Val - valine; W, Trp- tryptophan; Y, Tyr - tyrosine; and p.Y., P.Tyr - phosphotyrosine.

I. Nucleic Acid Molecules of the Invention

25 As hereinbefore mentioned, the present invention provides a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpA protein, or a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpB protein. The term "isolated and purified" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when
30 chemically synthesized. An "isolated and purified" nucleic acid is also free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

In an embodiment of the invention, a nucleic acid molecule is provided which encodes
35 TbpA having the amino acid sequence as shown in Figure 22 or SEQ.ID.NO:2. In another embodiment, a nucleic acid molecule is provided which encodes TbpB having the amino acid sequence as shown in Figure 24 or SEQ.ID.NO:4. In preferred embodiments of the invention, the

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nucleic acid molecule is a DNA comprising the nucleotide sequence as shown in Figure 21 or SEQ.ID.NO:1, or the nucleotide sequence as shown in Figure 23 or SEQ.ID.NO:3.

The invention includes nucleic acid sequences complementary to the nucleic acid (a) encoding TbpA having the amino acid sequence as shown in Figure 22 or SEQ.ID.NO:2; (b) encoding TbpB having the amino acid sequence as shown in Figure 24 or SEQ.ID.NO:4; (c) having the sequence as shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3. Preferably, the sequences are complementary to the full length nucleic acid sequences sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3.

The invention also includes nucleic acid molecules having substantial sequence identity or homology to the nucleic acid sequence as shown in Figure 21 or SEQ.ID.NO:1 or Figure 23 or SEQ.ID.NO:3; or encoding TbpA or TbpB proteins having substantial homology to the amino acid sequences shown in Figure 22 or SEQ.ID.NO:2, or in Figure 24 or SEQ.ID.NO:4, respectively. Homology refers to sequence similarity between sequences and can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same nucleotide base or amino acid, then the molecules are matching or have identical positions shared by the sequences.

Nucleic acid sequences having substantial homology include (a) nucleic acid sequences having at least 40-60%, preferably 60-80% most preferably 80-90% identity with the nucleic acid sequence as shown in Figure 21 or SEQ.ID.NO:1; and (b) nucleic acid sequences having at least 40-60%, preferably 60-80% most preferably 80-90% identity with the nucleic acid sequence as shown in Figure 23 or SEQ.ID.NO:3.

Another aspect of the invention provides a nucleic acid molecule, and fragments thereof having at least 15 nucleotide bases, which hybridizes to the nucleic acid molecules of the invention under hybridization conditions, preferably stringent hybridization conditions. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or may be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

Isolated and purified nucleic acid molecules encoding a protein having the activity of TbpA or TbpB, and having a sequence which differs from the nucleic acid sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, respectively, due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids should encode functionally equivalent TbpA or TbpB proteins but differ in sequence from the sequence in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, respectively, due to degeneracy in the genetic code.

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An isolated and purified nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of the nucleic acid sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, and using the labelled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated and purified nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding TbpA or TbpB using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding TbpA or TbpB into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein which exhibits TbpA or TbpB activity.

A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a protein having TbpA or TbpB activity can be accomplished by expressing the DNA in an appropriate host cell by standard techniques, and testing the ability of the expressed protein to bind ruminant transferrins and/or mediate iron uptake. A cDNA having such activity can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

Regulatory elements of *tbpA* or *tbpB* can be identified using conventional techniques. The function of the elements can be confirmed by using these elements to express a reporter gene

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which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures.

The sequence of a nucleic acid molecule of the invention may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An
5 antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

II. Recombinant TbpA and TbpB

The invention also contemplates a purified and isolated TbpA or TbpB protein from *Pasteurella haemolytica* A1 which exhibits transferrin binding activity. In an embodiment of
10 the invention, a purified TbpA protein is provided which has the amino acid sequence as shown in Figure 22 or in SEQ. ID. NO:2. In another embodiment of the invention, a purified TbpB protein is provided which has the amino acid sequence as shown in Figure 24 or in SEQ. ID. NO:4. Recombinant TbpB, unlike the native receptor complex, recognizes binding determinants on the N-lobe and C-lobe of transferrin.

In addition to the full length TbpA or TbpB amino acid sequences, the proteins of the present invention include truncations of TbpA or TbpB, and analogs, and homologs of TbpA or TbpB and truncations thereof as described herein. Truncated proteins may comprise peptides with at least 3 amino acid residues. The truncated proteins may have an amino group (-NH₂), a hydrophobic group (for example, carbobenzoxy, dansyl, or T-butyloxycarbonyl), an acetyl
20 group, a 9-fluorenylmethoxy-carbonyl (PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated proteins may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of TbpA or TbpB as shown in Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4, respectively, and/or truncations thereof as described herein, which may include, but are not limited to TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or
30 non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the TbpA or TbpB amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to TbpA or TbpB. Non-conserved substitutions involve replacing one or more amino acids of the TbpA or TbpB amino acid sequence with one or more
35 amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4). Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length.

Deletions may consist of the removal of one or more amino acids, or discrete portions from the TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), sequence. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 100 amino acids.

5 The proteins of the invention also include homologs of TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), and/or truncations thereof as described herein. Such TbpA or TbpB, homologs are proteins whose amino acid sequences are comprised of the amino acid sequences of TbpA or TbpB regions from other species that hybridize under stringent hybridization conditions (see discussion of stringent hybridization conditions herein)
10 with a probe used to obtain TbpA or TbpB.

Protein sequences having substantial homology include protein sequences having at least 40-60%, preferably 60-80%, most preferably 80-90% identity with the amino acid sequence as shown in Figure 22 (or SEQ. ID. NO:2) or Figure 24 (SEQ. ID. NO:4).

15 The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein.

The present invention also includes a TbpA, TbpB or aTbpA or TbpB related proteins conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion
20 proteins. Additionally, immunogenic portions of TbpA, TbpB or aTbpA or TbpB related proteins are within the scope of the invention.

TbpA, TbpB or a TbpA or TbpB related protein of the invention are prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which encodes TbpA, TbpB, or a TbpA or TbpB related protein of the
25 invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. The expression vectors are "suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that
30 the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted
35 protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art.

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The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein which confers resistance to certain drugs, or β -galactosidase.

5 The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification.

Recombinant expression vectors can be introduced into host cells to produce a
10 transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Suitable methods for transforming and
15 transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl,
20 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

N-terminal or C-terminal fusion proteins comprising TbpA, TbpB, or a TbpA or TbpB related protein of the invention conjugated with other molecules, such as proteins, may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of TbpA, TbpB or a TbpA or TbpB related protein, and the sequence of a selected protein or selectable
25 marker protein with a desired biological function. The resultant fusion proteins contain TbpA, TbpB or a TbpA or TbpB related protein, fused to the selected protein or marker protein.

III. Applications of the Invention

The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences in samples. Suitable probes
30 include nucleic acid molecules based on nucleic acid sequences encoding at least 6 sequential amino acids from regions of the TbpA or TbpB protein as shown in Figure 22 and SEQ. ID. NO: 2, or in Figure 24 and SEQ. ID. NO: 4, respectively. For example, a suitable probe may include nucleic acid molecules of TbpA selected from nucleotide nos. 1741 to 2784 of the sequence of TbpA shown in Figure 21 and SEQ ID NO:1. A nucleotide probe may be labelled with a detectable
35 substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P , ^3H , ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label

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may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic acid probes may be used to detect genes, preferably in human cells, that encode TbpA, TbpB, or TbpA or TbpB related proteins.

TbpA, TbpB, or TbpA or TbpB related proteins of the invention can be used to prepare antibodies specific for the proteins. Conventional methods can be used to prepare the antibodies. To produce polyclonal antibodies a mammal (such as a rabbit, mouse or rat) may be immunized with TbpA, TbpB, fragments of the proteins or a mixture of the two. The immunogenicity of the protein(s) may be enhanced by adding an adjuvant to the protein mixture or by coupling the protein to an immunogenic carrier. Examples of carriers include keyhole limpet hemocyanin(KLH) and bovine serum albumin(BSA).

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an animal (immunized as described above) and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, [e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989))]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for TbpA or TbpB, or TbpA or TbpB related proteins, as described herein.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a protein, or peptide thereof, having the activity of TbpA or TbpB. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Multivalent antibodies may be prepared by fusing two or more F(ab')₂ or Fab' fragments. For example, a multivalent antibody may contain one F(ab')₂ fragment specific for TbpA and one F(ab')₂ fragment specific for TbpB.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-ruminant animal variable region and a ruminant constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen

binding domain from an antibody of a mouse, rat, or other species, with bovine constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of novel *Tbp* genes of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature
5 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494.

Antibodies specifically reactive with TbpA, TbpB, or TbpA or TbpB related proteins, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used as probes to detect TbpA, TbpB, or TbpA or TbpB related proteins in samples such as tissues and cells, for
10 example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of TbpA, TbpB, or TbpA or TbpB related proteins and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g.ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. Thus, the antibodies may be used to detect and quantify TbpA, TbpB, or
15 TbpA or TbpB related proteins in a sample. In an embodiment, the antibodies are cross reactive against TbpA or TbpB or TbpA, or TbpB related proteins, from a wide range of serotypes of *P. haemolytica*. When used as probes the antibodies are usually labelled by techniques known in the art.

The antibodies of the present invention may also be used in the diagnosis and
20 treatment of *P. haemolytica* infection. In one embodiment, the antibodies are used in passive immunization to treat or prevent diseases in ruminants caused by *P. haemolytica*. In such a case, a mixture of antibodies or a multivalent antibody may be used.

The invention still further provides a method for identifying a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form
25 thereof, comprising reacting TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, with at least one substance which potentially can bind with TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, and assaying for complexes, for free substance, for
30 non-complexed TbpA or TbpB or a TbpA or TbpB related proteins or an activated form thereof. Substances which potentially can bind TbpA or TbpB, or TbpA or TbpB related proteins, include transferrins, particularly ruminant transferrins, analogs and derivatives of transferrins and antibodies against TbpA and TbpB, or TbpA or TbpB related proteins.

Still further, the invention provides a method for assaying a medium for the
35 presence of an agonist or antagonist of the interaction of TbpA or TbpB, or TbpA or TbpB related proteins, and a substance which binds to TbpA or TbpB, or TbpA or TbpB related proteins or an activated form thereof. In an embodiment, the method comprises providing a known concentration of TbpA or TbpB, or TbpA or TbpB related proteins, with a substance which is

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capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins and a suspected agonist or antagonist substance under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB, or TbpA or TbpB related proteins. In a
5 preferred embodiment of the invention, the substance is a ruminant transferrin, analog, derivative or part thereof, or an antibody against TbpA or TbpB, or TbpA or TbpB related proteins.

Substances which affect expression of TbpA or TbpB, or TbpA or TbpB related proteins, may also be identified using the methods of the invention by comparing the pattern
10 and level of expression of TbpA or TbpB, or TbpA or TbpB related proteins of the invention, in cells in the presence, and in the absence of the substance.

The substances identified using the methods of the invention may be used in the treatment of animals, particularly ruminants infected with *P. haemolytica* and accordingly they may be formulated into pharmaceutical compositions for administration to ruminants, such
15 as cattle, sheep and goats suffering from infection with *P. haemolytica*, or exposed to infection by *P. haemolytica*.

The substances may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the
20 substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may
25 vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or
30 rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects,
35 such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not

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exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

5 TbpA or TbpB, and/or TbpA or TbpB related proteins may be used as vaccines for the prophylaxis and treatment of various infectious diseases in animals. Infectious diseases contemplated by the invention include infections caused by *P. haemolytica*, such as bovine pneumoniae in cattle and systemic disease and pneumonia in sheep. In addition, vaccines according to the present invention may be used for the prophylaxis and treatment of infections caused by other *Pasteurella* spp. One example is the prophylaxis and treatment of *Pasteurella*
10 *multocida* in cattle, swine and poultry including respiratory and systemic infections such as Hemorrhagic septicemia and bovine mastitis. The vaccines can be intended for administration to various animals, preferably ruminants, including cattle, sheep, and goats.

The present inventors have demonstrated that different strains of *P. haemolytica*, from a range of ruminants, are able to bind and utilize a range of ruminant transferrins. Thus it is
15 contemplated that the vaccine compositions of the invention will be useful as broad spectrum vaccines suitable for immunizing a range of ruminants, such as sheep, cows and goats against infection with a wide range of *P. haemolytica* biotypes and serotypes.

The vaccine compositions comprise the TbpA, TbpB, and/or TbpA or TbpB related proteins, either alone, or in combination. The vaccine compositions may contain any combination
20 of the described proteins or immunogenic fragments thereof. Further, the composition may contain Tbp proteins isolated from one or more biotypes or serotypes of *P. haemolytica* or other microorganisms. Recombinant proteins comprising TbpA, TbpB, and/or TbpA or TbpB related proteins are preferably employed in the vaccine compositions of the invention. In a preferred embodiment, one or more of recombinant TbpA or TbpB, and TbpA or TbpB related proteins of the
25 invention, are used in the vaccine compositions. In another embodiment of the invention, the vaccine composition consists of purified and isolated TbpA and TbpB, preferably recombinant TbpA and TbpB.

The vaccine of the invention contains an immunologically effective amount of one or more of TbpA, TbpB, TbpA related protein, and TbpB related protein. The optimum amounts of
30 the proteins depends on the nature of the infection against which protection is required, the characteristics of the animals to be protected, and other factors known to persons skilled in the art.

In addition to the TbpA, TbpB, TbpA related protein, and/or TbpB related protein, the vaccine may comprise an immunologically acceptable carrier such as aqueous diluents,
35 suspending aids, buffers, excipients, and one or more adjuvants known in the art. Suitable adjuvants include aluminum hydroxide, Freund's adjuvant (complete or incomplete), bacteria such as *Bordetella pertussis* or *E. coli* or bacterium derived matter, immune stimulating complex (iscom), oil, saponin, oligopeptide, emulsified paraffin-Emulsigen™ (MVP Labs, Ralston,

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Nebraska), L80 adjuvant containing $Al(OH)_3$ (Reheis, New Jersey), Quil A (Superphos), or other adjuvants known to the skilled artisan. Preferably, the adjuvant is L80 adjuvant containing $Al(OH)_3$ (Reheis, New Jersey) and Quil A (Superphos). The vaccine can be incorporated into a liposome system which will allow the slow release of the TbpA and/or TbpB protein in the recipient. The vaccine may also contain preservatives such as sodium azide, thimersol, gentamicin, neomycin, and polymyxin.

The vaccine may be a multivalent vaccine and additionally contain other immunogens of *P. haemolytica* or immunogens related to other diseases in a prophylactically or therapeutically effective manner. For example, the vaccine composition of the invention may consist of *P. haemolytica* leukotoxin and TbpB.

The vaccines of the invention may be administered in a convenient manner, such as intravenously, intramuscularly, subcutaneously, intraperitoneally, intranatally or orally. Preferably the vaccine is administered intramuscularly or subcutaneously.

The dosage will depend on the nature of the infection, on the desired effect and on the chosen route of administration, and other factors known to persons skilled in the art.

The invention also contemplates the use of recombinant viral vector vaccines and recombinant bacterial vector vaccines containing nucleic acid molecules of the invention encoding TbpA, TbpB, TbpA related protein, and/or TbpB related protein, for the treatment and/or prophylaxis of *P. haemolytica* infection. In such systems, the TbpA or TbpB proteins are synthesized *in vivo* in the recipient from the exogenous nucleic acid molecules in the vaccine. The recombinant viral or bacterial vectors may be constructed using techniques known in the art, and as described herein. Examples of bacterial systems include *E. coli* and *Salmonella spp.*

The following non-limiting examples are illustrative of the present invention:

Examples

EXAMPLE 1

The following materials and methods were used in the studies described in the example:

MATERIALS AND METHODS

BACTERIAL STRAINS AND CLONING VECTORS

P. haemolytica strains were provided by Dr. P. Shewen, Department of Veterinary Microbiology and Immunology (VMI), University of Guelph, and were originally obtained from Dr. E. Biberstein, University of California, Davis, Dr. G. Frank, USDA, Ames, Iowa, and Dr. W. Donachie, Moredun Research Institute, Edinburgh, U.K. *Actinobacillus suis* strain 3714, *A. pleuropneumoniae* strains CM5 and Shope 4074 were provided by Dr. S. Rosendal, VMI. *E. coli* strains HB101 and TG-1 were provided by Dr. R. Lo, Department of Microbiology, University of Guelph, and were used as recipient strains for cloning experiments. *E. coli* strain JM109 (DE3) was provided by Dr. C. Whitfield, Department of Microbiology, University of Guelph.

Pasteurella and *Actinobacillus* strains were maintained on sheep's blood agar and cultured in brain heart infusion broth (BHIB), (Difco Labs, Detroit, Michigan). *E. coli* HB101

was grown on Luria-Bertaini plus thymidine (LT), supplemented with ampicillin (Sigma Chemical Co., St. Louis, Missouri) at 100 mg/L for selection of recombinant plasmids. Similarly, *E. coli* TG-1 and JM109 (DE3) were grown on Davis minimal medium with ampicillin. Iron-depleted conditions were prepared by adding the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic) acid (EDDA) (Sigma) to a final concentration of 100 μ M. Iron-depleted conditions were prepared by adding FeCl₃ to 1 mM.

The plasmid pBR322, bacteriophage vectors M13/mp18 and M13/mp19 were used as previously described (Lo and Cameron, 1986; Lo *et al.*, 1985; and Lo *et al.*, 1987). The pBluescript vector was obtained from Stratagene (La Jolla, California). The recombinant Clone 482 was provided by Dr. A. Schryvers, Department of Microbiology, University of Calgary.

ENZYMES, CHEMICALS AND ANTISERA

Restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories (BRL) (Burlington, Ontario) or Pharmacia Chemicals Incorporated (Dorval, Quebec) and were used as described by the manufacturers. Radioisotopes were purchased from ICN Biomedical (Montreal, Quebec) or Amersham Laboratories (Oakville, Ontario).

Goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and immunodetection reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario). Goat anti-bovine immunoglobulin G-Alkaline phosphatase conjugate was purchased from Jackson Immunoresearch (West Grove, PA). Rabbit anti-autologous antiserum and bovine anti-Prepsone antisera were obtained from Dr. P. Shewen, Department of Veterinary Microbiology and immunology, University of Guelph. The rabbit "anti-autologous" antiserum was raised against the soluble antigens of *P. haemolytica* A1 cultured in RPMI 1640 supplemented with that rabbit's own serum. It is important to note that RMPI 1640 is an iron poor medium.

DNA METHODS

a) Chromosomal DNA isolation

Chromosomal DNA was isolated from bacterial cells according to the method of Marmer (1961). Bacteria were inoculated into 250 ml of the appropriate medium and grown overnight at 37°C with 150 rpm shaking. The following day, the cells were pelleted by centrifugation at 4,000 \times g in a GSA rotor in a Sorvall RC5-B refrigerated centrifuge (Dupont Instruments, Mississauga, Ontario) for 10 min. The pellet was suspended in 8 ml of a 0.6 M sorbitol, 0.05 mM Tris-HCl (pH 8.0), 0.05 M EDTA solution. Lysozyme (Sigma) was added to a final concentration of 3 mg/ml and the sample was incubated for 30 min on ice. Two ml of lytic solution (0.5% SDS, 0.05 M EDTA, 0.05 M Tris-Cl [pH 8.0]) and 3 mg/ml of proteinase K (Sigma) solution were added to the sample, which was then incubated for 4 h in a 37°C water bath, followed by incubation at 56°C.

The suspension was extracted with an equal volume of phenol (Gibco/BRL) saturated with TE buffer (0.05 M Tris-HCl [pH 7.5], 0.001 M EDTA) and shaken at 30-50 rpm for 45 min.

The phenol and aqueous phases were separated by centrifugation at $12,000 \times g$ at 5°C for 10 min in a SS34 rotor. The supernatant was collected by a cut off wide mouth pasteur pipette and the DNA was precipitated with 2-3 volumes of ice-cold 95% ethanol. The strands of DNA were spooled onto a glass rod and dissolved in a small volume of 0.1 X SSC (1X SSC contains 0.15 M NaCl, 0.015 M sodium citrate).

The DNA was then treated with RNase to a final concentration of $10\mu\text{g/ml}$ and incubated at 37°C for 30 min. The DNA was again precipitated by 2-3 volumes of cold 95% ethanol, spooled onto a glass rod and dissolved in 1X SSC. Samples were stored at 4°C .

b) Restriction endonuclease digestion and ligation

Plasmid and bacteriophage vectors were digested with the appropriate restriction endonucleases according to manufacturer's instructions. Vector and insert DNA were mixed to a final volume of $5\mu\text{l}$ and were ligated with 0.5 units of T4 DNA ligase. Ligation mixtures were either incubated for 3-4 hours at room temperature, or overnight at 14°C prior to transformation into *E. coli* cells.

c) Preparation of competent *E. coli* cells

Transformation was used to introduce plasmid and bacteriophage DNA into competent *E. coli* cells (Mandel and Higa, 1970; Lederberg and Cohen *et al.*, 1972). *E. coli* strains to be transformed were grown overnight in LT broth at 37°C with 150 rpm shaking. The following day, a 1/40 subculture in 20 ml of the same medium was prepared and grown for an additional 60 min at 37°C with 75 rpm shaking. The cells were collected by centrifugation at $3,000 \times g$ in an SS34 rotor and resuspended in 10 ml sterile ice-cold 50 mM CaCl_2 . The suspension was incubated for 30 min. on ice, then the cells were collected by centrifugation and resuspended in 2 ml sterile ice-cold mM CaCl_2 . The competent cells could then be stored at 4°C and used for up to 3 days.

For transformation, 0.2 ml of the competent cells were mixed with the DNA sample and incubated for 30 min on ice. The cells were heat-shocked for 2 min at 42°C and then 0.2 ml of LT broth was added. The cells were incubated at 37°C for 15 min then plated onto LT plates containing appropriate antibiotics and incubated overnight at 37°C .

d) Large-scale plasmid isolation

Large-scale plasmid isolation was performed according to the procedure of Clewell and Helinski (1969) with modifications. *E. coli* carrying the plasmid was inoculated into 250 ml LT broth containing ampicillin and grown overnight at 37°C with 150 rpm shaking. The following day, chloramphenicol (Sigma) was added to a final concentration of 25 mg/l and the culture was grown for a further 4-6 h. The cells were collected by centrifugation at $4,000 \times g$ for 10 min in a GSA rotor. The cell pellet was resuspended in 4 ml of an ice-cold solution containing 25% sucrose and 0.05 M Tris HCl (pH 8.0), then 1 ml of a fresh 10 mg/ml lysozyme (Sigma) solution was added. The mixture was incubated in a 37°C waterbath for 30 min, placed on ice for 5 min and then 2 ml of 0.25 M EDTA (pH 8.0) was then added. After a further 5 min incubation on ice, 5

ml of a lytic solution (0.05 M Tris-HCl [pH 8.0], 0.0625 M EDTA and 2% Triton X-100) were added. The mixture was returned to the 37°C waterbath for 5-15 min until cell lysis was complete. The mixture was then centrifuged for 30 min at 27,000 x g and the clear lysate was transferred to a clean test tube, the lysate was mixed with solid CsCl (Boehringer Mannheim, Laval, Quebec) at 1 g/ml to a total of 4.5 ml. One hundred µl of ethidium bromide (10 mg/ml) were then added in 4.5 ml of the sample. The centrifuge tube was heat sealed and the sample was centrifuged at 240,000 x g for a minimum of 9 h at 15°C in a Beckman VTi65 vertical rotor.

Plasmid DNA was recovered by puncturing the top and bottom of the centrifuge tube and collecting the lower of two bands in the tube. To extract the ethidium bromide from the sample, the plasmid DNA solution was mixed with an equal volume of Cs/Cl saturated n-butanol. After allowing the phases to separate, the upper layer containing n-butanol and ethidium bromide was removed. This process was repeated three times. Following ethidium bromide extraction, the lower aqueous phase was dialyzed to remove the CsCl. Dialysis tubing (Fisher) with a molecular cutoff of 10 kDa was prepared by boiling 2 x 15 min in 0.1 M Na bicarbonate and 1 x 15 min in 0.25 M EDTA (pH 7.5) and was stored at 4°C in 50% ethanol and 1mM EDTA. Prior to dialysis, the tubing was rinsed in dH₂O and then filled with the plasmid DNA solution. The DNA was dialyzed for 24 h in 4 x 1L of dialysis buffer (0.01 M Tris-HCl [pH 7.5 at 4°C], and 0.001 M EDTA) at 4°C. The sample was stored at -20°C.

Alternatively, the Flexi-prep kit from Pharmacia (Quebec City, Quebec) was used for small-scale plasmid preparation. This method involved a standard alkaline cell lysis, including RNase treatment and isopropanol precipitation (Birnboim and Doly, 1979; Isch-Horowicz and Burke, 1981). The plasmid DNA was purified and concentrated using a silica matrix (Sephaglas FPTM) in guanidine hydrochloride.

e) **Radiolabelling of DNA probes by random priming**

DNA fragments were labelled with [α -³²P] dATP (3,000 Ci/mmol, ICN) using the random primer DNA labelling system of GIBCO/BRL. This labelling system is based on the method of Feinberg and Vogelstein (1983), with modifications (Feinberg and Vogelstein, 1984). The sample (25 ng of DNA in 10 µl of H₂O) was denatured by boiling for 5 min, then immediately cooled on ice. While still on ice, the following reagents were added: 2µl of each of dCTP, dGTP and dTTP, 15 µl of random primer buffer, 4 µl [α -³²P]dATP and H₂O to 49µl. The sample was mixed briefly and 3 units of Klenow Fragment was added. The reaction mixture was incubated for 1 h at 25°C and terminated by the addition of 5 µl of stop buffer.

The radiolabelled DNA was separated from unincorporated radionucleotides by gel filtration through a mini Sephadex G-50 column. The column was prepared in a Pasteur pipette plugged with glass wool and was equilibrated with TE buffer prior to addition of the radiolabelled sample. The migration of DNA through the column was monitored using a Geiger counter (Mini-Instruments Ltd., Essex, England). The first peak of radioactivity corresponded to

the labelled DNA, while the second peak corresponded to the unincorporated [³²P]-dATP. The DNA probe was denatured by boiling for 5 min before being added to the hybridization solution.

f) **Agarose gel electrophoresis and Southern hybridization**

Agarose gels were prepared by adding TAE buffer (40 mM Tris [pH 7.9], 1mM EDTA) to electrophoresis grade agarose powder (regular or low-melting point; Sigma) to a final concentration of 0.7% to 1%. The agarose gel was electrophoresed in a horizontal flatbed gel apparatus (Tyler Research, Edmonton, Alberta).

DNA samples were mixed with 1/2 volume of tracking dye (50% glycerol, 0.1% ladder (Gibco/BRI), or lambda DNA (Pharmacia) digested with *Hind*III was used as a molecular standard. A running buffer of TAE supplemented with 1 µg/ml of ethidium bromide was used. Samples were initially electrophoresed at 100V for 5 min, then the voltage was reduced to 10-12V for overnight electrophoresis. After electrophoresis, the samples were viewed with a medium range ultraviolet transilluminator and photographed using Polaroid type 57 black and white film (Sharp *et al.*, 1973; Hayward, 1972).

For Southern hybridization, the agarose gel was immersed in 0.25 M HCl for 15 min to depurinate the DNA. The gel was transferred to an alkaline solution consisting of 0.5 M NaOH and 1.5 M NaCl for 15 min, then neutralized in a solution of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30 min. The DNA was transferred to a nitrocellulose membrane (Schleicher and Shuell, Willowdale, Ontario) by electrophoretic transfer in a semi-dry blotting apparatus (Tyler Research) in 20X SSPE buffer (3.6 M NaCl, 0.2 M Na₂PO₄ [pH 7.0], 0.02 M Na₂EDTA, 0.16 M NaOH) at a constant current of 150 mA for 30 minutes (Wahl *et al.*, 1979; Southern, 1975).

After electrophoretic transfer, the nitrocellulose membrane was washed in 2X SSPE buffer for 10 min, and the DNA was cross-linked by a UV Cross-linker (Stratagene). The membrane was prehybridized in a sealed plastic bag containing a solution of 25% (low stringency) or 50% (high stringency) formamide (Gibco/BRL) in 0.1% glycine, 5X BFP (100X BFP contains 2% w/v bovine serum albumin, Ficoll and polyvinyl pyrrolidone-40), 5X SSPE buffer and 0.1 mg/ml sonicated, boiled salmon sperm carrier DNA. The sealed bags were placed in a 42°C shaking waterbath where the membranes were allowed to prehybridize for at least an hour. The prehybridization buffer was then discarded and replaced with hybridization buffer (10% dextran sulphate, 5X SSPE, 5X BFP, 0.1% SDS, 0.1 mg/ml carrier DNA and 25% or 50% formamide) containing the boiled, radiolabelled DNA probe. The bags were placed in a 42°C shaking waterbath where the membranes were hybridized overnight.

After hybridization, the nitrocellulose membrane was removed from the plastic bag and washed 4 x 10 min in either high stringency (5X SSPE, 0.1% SDS) or low stringency (2X SSPE, 0.1% SDS) wash buffer in a 42°C shaking waterbath. The membrane was air-dried, placed on Whatman filter paper, covered with plastic wrap and exposed to X-ray film (Cronex, Willingmington, Delaware) at -20°C for 104 days until the desired exposure was obtained. The exposure time was determined by measuring the intensity of the radioactive signal using a

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Geiger counter. Autoradiographs were developed in Kodak GBX rapid developer (Eastman Kodak, Rochester, New York) for 2 min. The developing reaction was stopped by immersing the film in 2.5% acetic acid for 1 min and fixed for 2 min in Kodak GBX fixer (Eastman Kodak).

g) Southern colony blot

5 A master template of bacterial colonies grown on LT plus ampicillin was grown overnight at 37°C. The colonies on the master plate were duplicated onto a nitrocellulose membrane overlaid on an LT plus ampicillin plate and grown for 2-3 h at 37°C. The membrane was then overlaid on Whatman filter paper soaked in a 0.5 M NaOH, 1.5 M NaCl solution and incubated at room temperature (RT) for 5 min to lyse the cells. The nitrocellulose membrane was
10 then transferred to Whatman filter paper soaked in a 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl solution and incubated for 5 min at RT to neutralize the membrane. The membrane was transferred to Whatman filter paper soaked in 95% ethanol and sprayed with 95% ethanol to precipitate the DNA. The DNA on the membrane was cross-linked in a UV cross-linker (Stratagene), then prehybridized and hybridized as described above.

15 h) Polymerase chain reaction (PCR)

PCR reactions were carried out in thin-walled 500 µl GeneAmp microfuge tubes in a Perkin-Elmer Cetus 480 DNA Thermal Cycler, using the Perkin-Elmer Cetus PCR core reagent kit which included deoxynucleotides triphosphates, MgCl₂, reaction buffer and Ampli-Taq DNA polymerase (Perkin-Elmer Cetus). Amplification reactions were performed according to the
20 method of Saiki *et al.* (1988), with modifications by Perkin-Elmer Cetus. PCR reactions were performed in 100 µl mixtures containing 1X reaction buffer (0.5 M KCl, 0.1 M Tris-HCl [pH 9.0]), 0.2 mM of each of dNTP, 0.4 µM primer, 5 µg of template, 15 mM MgCl₂ and 2.5 units of Ampli-Taq enzyme. The reaction mixture was heated at 95°C for 2 minutes to denature the template DNA. Then 30 cycles of denaturation, annealing and extension followed with temperatures and
25 times of 95°C (1 min) 52°C (1 min) and 72°C (2 min) respectively. The fastest available transitions between temperatures (ramp time of 0.01s) were used. A negative control which did not contain template DNA was included in each PCR run.

After amplification, the PCR products were examined by agarose gel electrophoresis. PCR products were purified by electrophoretic separation through a low-
30 melting point agarose gel followed by excision of required DNA fragments. The DNA products were purified from the agarose using a glass-bead matrix purification kit (GENECLEAN).

i) Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using the GENECLEAN kit from Bio/Can Scientific (Mississauga, Ontario). The GENECLEAN purification process is based on
35 the procedure by Vogelstein and Gillespie (1979). The gel slice containing the fragment was excised from the gel using a razor blade and placed in a 1.5 ml Eppendorf centrifuge tube. An equal volume of stock NaI solution was added and the sample was incubated for 5 min in a 55°C waterbath until the agarose had completely melted.

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GLASSMILK (Bio/Can Scientific) was added to the sample at a volume of 5 µl for 5µg or less of DNA and the mixture was incubated on ice for 5 min. The silica matrix was then collected by centrifugation at 16,000 x g for 10 sec and resuspended in 600 µl of NEW wash buffer (Bio/Can Scientific). The pellet was washed with NEW buffer a total of three times. After the
5 final wash, the silica matrix was resuspended in 10µl of TE buffer and incubated for 5 min at 55°C. The sample was then centrifuged and the TE recovered, avoiding the silica matrix pellet. Samples were stored at -20°C.

j) **DNA dideoxy sequencing**

DNA fragments were sequenced either by cloning into M13 mp18/mp19 bacteriophage
10 vectors (single-stranded sequencing) or directly from recombinant plasmids (double-stranded sequencing) using the Pharmacia T7-sequencing kit as described by the manufacturer. The Pharmacia T7-sequencing kit procedure is based on the method outlined by Sanger *et al.* (1977).

For single stranded sequencing, DNA fragments were cloned into the M13 mp18/mp19 bacteriophage vector and transformed into competent *E. coli* TG-1 cells.
15 Recombinant phage "plaques", which appeared white due to the loss of β-galactosidase production, were selected. Each plaque was inoculated into 10 ml of LT broth seeded with 0.1 ml overnight culture of *E. coli* TG-1 grown in Davis minimal medium and incubated 4-5 h at 37°C with 75 rpm shaking. The sample was centrifuged at 12,000 x g for 10 min to remove the *E. coli* cells. The phage were precipitated from the culture supernatant by the addition of 1/4 volume
20 of 20% polyethylene glycol (8,000 MW; Sigma), 2.5 M NaCl and incubated for 30 min on ice. Precipitated phage were recovered by centrifugation in a microfuge at 12,000 x g for 10 min. The pellet was then resuspended in 0.6 ml of phage buffer (0.1 M Tris-HCl [pH 8.0], 0.001 M EDTA, 0.3 M NaCl).

The phage DNA was extracted with 0.5 ml of phenol (Gibco/BRL) saturated with
25 TE buffer. The phenol and aqueous phases were separated by centrifugation at 14,000 x g for 10 min. The aqueous phase was extracted with 1:1 phenol:chloroform and finally with chloroform. The phage DNA was then precipitated with 1/10 volume 3 M sodium acetate (pH 7.0) and 2 volumes of cold 95% ethanol and incubated at -20°C overnight. Precipitated DNA was collected by centrifugation at 14,000 x g for 10 min. The aqueous phase was extracted with 1:1
30 phenol:chloroform and finally with chloroform. The phage DNA was then precipitated with 1/10 volume 3 M sodium acetate (pH 7.0) and 2 volumes of cold 95% ethanol and incubated at -20°C overnight. Precipitated DNA was collected by centrifugation at 14,000 x g for 10 min in a 4°C microfuge. The pellet was air-dried and resuspended in 50 µl of TE buffer and was used for sequencing. The DNA was annealed to either the universal M13 primer or specific primers in the
35 presence of annealing buffer (Pharmacia T7 sequencing kit) by incubation at 65°C for 10 min, then room temperature for 10 min prior to the sequencing reactions.

For double-stranded sequencing, the plasmid template was prepared using the procedure outlined in the Pharmacia T7 sequencing kit protocol, with modifications. Plasmid

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DNA was adjusted to 1.5-2.0 µg/32 µl and denatured by the addition of 12 µl of 2M NaOH for 1 min. Denaturation was terminated by the addition of 11 µl of 3 M sodium acetate (pH 5.0). The DNA was precipitated with 7 µl of dH₂O and 120 µl of ice-cold absolute ethanol and incubated at -20°C overnight.

5 The DNA was collected by centrifugation at 14,000 x g in a 4°C microfuge for 10 min. The pellet was washed with 100 µl of ice-cold 70% ethanol, centrifuged and dried under vacuum. The sample was resuspended in 5 µl of dH₂O and mixed with 5 µl of primer and 2 µl of annealing buffer. The mixture was incubated at 65°C for 5 min, 37°C for 10 min and 5 min at RT prior to sequencing.

10 Either [³²P]dATP or [³⁵S]dATP (specific activity of 3000 Ci/mmol) were used in the sequencing reactions. For short autoradiography exposure time, [³²P]dATP was used. For superior resolution, [³⁵S]dATP was used. Oligonucleotide primers were synthesized on an Applied Biosystems International 391 PCR-Mate DNA synthesizer and purified according to the manufacturer's instructions. The primers were quantitated by measuring the optical density
15 at 260 nm prior to use.

For [³²P]dATP sequencing, the sequencing gel consisted of 18 g of urea (ICN, Montreal, Quebec), 3.75 ml of 10X TBE buffer (1 M Tris [pH 8.3], 0.02 M EDTA, and 0.865 M boric acid) and 7.5 ml of 40% acrylamide (19:1 ratio of acrylamide:bisacrylamide, Bio-Rad) made up to 38 ml with dH₂O. The solution was stirred until the urea was dissolved and then 0.23 ml of 10%
20 ammonium persulfate (Sigma) and 10 µl of TEMED (N, N, N'-Tetramethylethylenediamine; Sigma) (0.01% final concentration) were added for polymerization.

For [³⁵S]dATP sequencing the gel consisted of 16.8 g of urea (ICN), 4.8 ml of 10X TBE buffer and 4 ml of a modified acrylamide solution ("Long Ranger", J.T. Baker, Phillipsburg, New Jersey). The solution was made up to 40 ml with dH₂O and polymerized with 200 µl of
25 ammonium persulfate (Sigma) and 20 µl of TEMED (Sigma).

The running buffer consisted of 1X TBE. The sequencing gel was run at 40 W/gel constant power for 2-6 hr. [³⁵S]dATP sequencing gels were transferred onto a sheet of Whatman filter paper and dried under vacuum at 80°C for 45 min. Both types of sequencing gels were exposed to Cronex 4 X-ray film (Cronex for 18-48 h at -20°C).

30 IV. PROTEIN METHODS

a) Isolation of inner and outer membranes

Inner and outer membrane preparations were prepared from *E. coli* and *P. haemolytica* A1 by the procedure of Hancock and Carey (1979), with modifications (Lo *et al.*, 1991). Bacteria were grown in 250 ml of the appropriate medium at 37°C overnight. The cells
35 were collected by centrifugation at 4,000 x g, washed twice in 0.01 M Tris-HCl (pH 6.8) and resuspended in 7.5 ml of a cold Sucrose-Tris solution containing 20% sucrose, 0.01 M Tris-HCl (pH 6.8), lysozyme (1mg/ml), DNase (50µg/ml), and RNase (100 µg/ml). The cells were lysed by

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French pressure cell three times at 16,000 - 18,000 psi at 4°C. The sample was then centrifuged at 1,085 x g for 5 min to remove unlysed cells.

The supernatant was layered onto a 70:52:sample:12% sucrose gradient which consisted of 14 ml of the 70 and 52% sucrose followed by 5 ml of the sample lysate and 4-5 ml of 12% sucrose. The gradient was centrifuged in a swinging bucket rotor at 80,000 x g for 16-18 h at 4°C. The inner and outer membrane fractions were collected by aspiration. The inner membrane fraction was located between the 12% and 52% sucrose regions and had a yellowish-brown colour. The outer membrane fraction, which was white, was located near the 70% sucrose region. The collected fractions were loaded into centrifuge tubes, topped up with dH₂O and centrifuged in a fixed-angle Ti80 rotor at 225,000 x g for 1 h at 4°C. The pellet was then air-dried and resuspended in a Tris-HCl (pH 6.8), 0.001 M dithiothreitol buffer. Samples were stored at -20°C.

b) Bradford determination of protein concentration

The protein concentrations of the inner and outer membrane fractions were determined using the method of Bradford (1976). Dilutions of each membrane fraction were prepared and dH₂O was added to a final sample volume of 0.8 ml. The sample was then mixed with 0.2 ml of Bradford reagent (Biorad, Mississauga, Ontario) and incubated at room temperature for 5 min to allow colour development to occur. The optical density (OD) of each sample was measured in a spectrophotometer at a wavelength of 595 nm. A standard curve was plotted using bovine serum albumin (BSA; Sigma) in the 1-25 µg range. The protein concentration of each sample was extrapolated from the BSA standard curve.

c) Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins were analyzed using sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) with 4% (w/v) stacking and 7.5% (w/v) separating gels (Laemmli, 1970). The gels were polymerized by the addition of 0.1% ammonium persulfate and TEMED to 0.01%. The samples were solubilized at 100°C for five minutes in an equal volume of 2X sample buffer. A high molecular weight standard was also boiled and loaded onto the gel. A discontinuous buffer system was used (0.192 M glycine, 0.02 M Tris-HCl [pH 8.4], 0.1% SDS).

The gels were run at 100V until the samples entered the separating gel, when the voltage was increased to 150V. The samples were run until the dye front ran off the bottom of the gel. The stacking gel was removed and the separating gel was either stained with Coomassie Brilliant Blue or electrophoretically transferred to nitrocellulose for Western immunoblotting. Gels were stained in Coomassie Brilliant Blue R250 (0.05% in 40% methanol, 10% acetic acid) (Eastman Kodak) overnight and then destained in a methanol: acetic acid solution.

d) Western immunoblot analysis

The proteins on the acrylamide gel were transferred to a nitrocellulose membrane according to the method of Burnette (1981). The gel was soaked in blotting buffer (0.192 M

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glycine, 0.025 M Tris-Cl [pH 8.4], 20% methanol) for 10 minutes to remove the SDS. A piece of nitrocellulose membrane (Schleicher and Shuell, Willowdale, Ontario) cut to fit the gel was also soaked in blotting buffer. The proteins were transferred to the nitrocellulose membrane in a Bio-Rad Transblot apparatus at 450 mA for 3 h. A water-cooling system was used to prevent heating and breakdown of the blotting buffer.

After electrophoretic transfer, the nitrocellulose membrane was soaked in 3% gelatin in TTBS buffer (0.02 M Tris-Cl [pH 7.5], 0.5 M NaCl, 0.05% Tween-20) for 30 min to block the membrane. The nitrocellulose membrane was transferred to a 1/500 dilution of the first antibody in 1% gelatin and incubated overnight at room temperature with gentle shaking. The membrane was then washed twice in TTBS buffer (15 min per wash) and placed in the second antibody solution (1/2000 dilution) for an hour. The second antibody was goat anti-rabbit or goat anti-bovine IgG-alkaline phosphatase conjugate (Bio-Rad) in 1% gelatin. The membrane was washed twice in TTBS buffer (15 min per wash) and then twice (5 min per wash) in NBT buffer (0.1 M Tris-Cl [pH 9.5], 0.1 M NaCl, 50 mM MgCl₂). The membrane was then placed in the developing solution of 100 µl of each of the reagents 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 25 mg/ml in dimethylformamide; Sigma) and nitro-blue-tetrazolium (NBT, 50 mg/ml in 70% dimethylformamide; Sigma). Colour development was allowed to proceed until the desired visibility of the bands was obtained. The colour reaction was stopped by rinsing the membrane in H₂O. The membrane was air-dried.

e) **T7 protein expression**

Proteins encoded by a recombinant plasmid were analyzed using the method of Tabor and Richardson (1985). The *thpA* gene was cloned into the plasmid vector pBluescript. The recombinant plasmid was transformed into *E. coli* JM109 (DE3), which is a strain of *E. coli* JM109 with the T7 RNA polymerase gene integrated into the chromosome and T7 polymerase gene under the control of the lac promoter (Yanisch-Perron *et al.*, 1985).

After transformation, the cells were grown overnight at 37°C in Davis minimal medium containing 1.0% casamino acids, 0.4% glucose and the appropriate antibiotics. A 1/50 subculture into 20 ml of the same medium was prepared and incubated at 37°C for an additional 3-4 h until the OD₅₅₀=0.6. The cells were collected by centrifugation at 14,000 x g for 5 min and the pellet was resuspended in Davis minimal medium with 0.4% glucose. The sample was incubated for 90 min at 37°C, then 100 µl of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added. After the cells were incubated at 37°C for 20 min, rifampicin was added (final concentration of 400 µg/ml). The sample was incubated at 37°C for 30 min and then labelled for 60 min with 5 µCi [³⁵S]-methionine ("Trans-Label", ICN Biomedical, Quebec). The cells were washed twice in ice-cold PBS and collected by centrifugation at 14,000 x g for 5 min in a microfuge. The pellet was resuspended in 2X SDS-PAGE sample buffer.

The proteins were separated using SDS-PAGE and the gel was stained with Coomassie blue R250. The gel was then soaked in Amplify™ (Amersham, Oakville, Ontario) for 30 min and dried under vacuum. Autoradiographs were exposed 18-48 h.

RESULTS

5 I. Preliminary Cloning of Putative *tbpA*, *tbpB* genes

The first stage in cloning the *tbpA* gene was to screen a *P. haemolytica* A1 gene library by polymerase chain reaction. An oligo primer specific for the N-terminal amino acid sequence of the Tbp1 protein was synthesized. A *P. haemolytica* A1 codon table (Lo, 1992) was used to optimize the primer sequence. The Tbp1 primer was used in conjunction with primers based on the junction sequences of the cloning vector pBR322 (Table 1). A 0.8 kbp PCR product was obtained (Figure 1) and cloned into the M13 vector and sequenced. Sequence analysis of this PCR product demonstrated that the first twenty amino acids matched the sequence obtained by N-terminal amino acid sequencing of Tbp1.

Table 1. Oligonucleotide primers used in PCR.

15	Primer	Primer Sequence	Size
	Tbp1	thr-glu-asn-lys-lys-ile-glu-glu 5'GGAAGCTTACT-GAA-AAT-AAA-AAA-ATC-GAA-GAA *	32 mer
	primer left	5' GGAATTCCTGCTGTGGATC **	22 mer
20	primer right	5' GTGAATTCGCGTAGAGGATC **	22 mer

* - the underlined sequence is the *Hind*III site

** - the underlined sequence is the *Eco*RI site

The 0.8 kb PCR product was then radiolabelled and used as a specific probe to screen the *E. coli* clones containing *P. haemolytica* A1 gene library by Southern hybridization. Two recombinant clones, 9 and 10, hybridized strongly with the *tbpA* probe. The plasmid DNA from each recombinant clone was analyzed by restriction endonuclease mapping (Figure 2). The insert of *P. haemolytica* A1 was determined to be approximately 8.7 kb and 2.3 kb for plasmids 9 and 10, respectively. Initial sequence analysis of the plasmids confirmed that the insert DNA from both plasmids share an overlapping region. Plasmid 9 contained the entire *tbpA* gene but the region directly upstream of *tbpA* was different than the upstream region in plasmid 10. It is possible that the insert DNA in plasmid 9 was formed from two DNA fragments from separate regions of the genome. In plasmid 10, the region directly upstream of *tbpA* contained an additional open reading frame which corresponded to the *tbpB* gene. This plasmid, therefore, contained not only the 5' region of *tbpA* but also part of the *tbpB* gene, directly upstream from the *tbpA* gene.

A third recombinant clone, 482, contained the entire *tbpB* gene. This plasmid shares an overlapping region with plasmid 10 (Figure 2). The insert DNA in plasmid 482 is a PCR product obtained from *P. haemolytica* A 1 genomic DNA using primers specific for the amino acid sequence of Tbp2 protein. This PCR product was then cloned into the vector PCRII.

The 3.0 kbp *tbpA* gene was sequenced from plasmid 9 (starting from the *Bgl*III site). The 2.1 kbp *tbpB* gene and the 91 bp sequence between *tbpA* and *tbpB* was sequenced from plasmids 482 and 10.

II. Sequence Analysis

5 The 5.2 kbp of DNA was sequenced and shown to contain two open reading frames arranged in tandem, with *tbpB* upstream of *tbpA* (Figure 3). This genetic organization is consistent with other iron uptake systems in other bacteria where the genes often arranged in an operon (Payne, 1988). Upon sequence analysis of the deduced Tbp1 protein, a putative 28 amino acid leader peptide was observed. A putative cleavage sequence for lipoproteins was observed
10 in the deduced Tbp2 protein. The close proximity of *tbpA* and *tbpB* and the absence of a promoter region in *tbpA* suggests that the two proteins may be coordinately expressed. A Fur consensus sequence in the promoter region of *tbpB* suggests that the proteins may be regulated in a Fur-like manner. The Fur consensus sequence in *P. haemolytica* *tbpB* is similar to the consenses sequence found in *N. gonorrhoeae* and *N. meningitidis* *tbpB* (Figure 4). The isoelectric point of Tbp1 and
15 Tbp2 was calculated by PCGene (Chargpro) to be 9.16 and 9.71 respectively, making them basic proteins.

III. Predicted Protein Topology

The sequence analysis program Gene Runner (Hastings Software) was used to analyze the physical characteristics and to predict the secondary structure of the *P. haemolytica* Tbp1
20 and Tbp2 proteins. The hydropathy plot of Tbp1 and Tbp2 were generated using the method of Kyte and Doolittle (1982). The first 28 amino acids of Tbp1 form a hydrophobic region, which is characteristic of all signal sequences. There are six other hydrophobic regions in the protein, which may be transmembrane domains of the protein. Hydrophilic areas of the protein may be either exposed at the cell surface or in the periplasm. The hydropathy plot of *N. gonorrhoeae*
25 was also generated. The *N. gonorrhoeae* Tbp1 protein seems to be less hydrophobic than *P. haemolytica* Tbp1 but the location of some of the hydrophobic regions are similar. For example, both proteins have hydrophobic regions around the 200, 400, and 780 amino acid residues. The similarity in hydrophobic regions suggests that the two proteins share a significant degree of homology and may have a similar structure.

30 The Kyte-Doolittle plot of *P. haemolytica* Tbp2 reveals several large hydrophobic regions in the centre of the protein and two smaller hydrophilic regions at each end. This is significantly different than the hydropathy plot of *N. gonorrhoeae* Tbp suggesting that the two proteins may have different structures.

35 Surface exposed regions of Tbp1 and Tbp2 were determined using the Emini surface probability method (Emini *et al.*, 1985). The peaks on the graph correspond to the regions with the highest probability of being exposed. Surface exposed regions of each protein may be involved in ligand binding and may be antigenic. The Emini plot of *P. haemolytica* Tbp1 suggests that the hydrophilic regions near amino acids 330, 410, 460, 560, 610 and 820 of the

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protein may be exposed at the cell surface. The Emini plot of *N. gonorrhoeae* Tbp1 showed a few common exposed regions with *P. haemolytica* Tbp1 (regions at 330, 580, 810).

The Emini plot of *P. haemolytica* Tbp2 suggests that the hydrophilic regions at amino acids 140, 160 and 620 have the greatest probability of being exposed. The Emini plot of *N. gonorrhoeae* Tbp2 shows that only the exposed regions at 160, 330 are similar to the surface regions of *P. haemolytica* Tbp2.

The secondary structure of both Tbp1 and Tbp2 were predicted using the method of Chou-Fasman (1978). The Chou-Fasman plot of *P. haemolytica* Tbp1 predicts that the protein is primarily a β -sheet and β -turn structure. The Chou-Fasman plot of *N. gonorrhoeae* Tbp1 predicts a similar structure. These predictions are consistent with other topology predictions of other iron-regulated outer membrane proteins, which are also composed of amphipathic β -sheets (Moeck *et al.*, 1994). It is also interesting to note that the location of the β -sheets correspond to the location of the hydrophobic domains in the two Tbp1 Kyte-Doolittle graphs.

The Chou-Fasman plot of *P. haemolytica* Tbp2 indicates that it also consists primarily of β -Sheets and β -turns. The predicted pattern of B-sheets is different than *N. gonorrhoeae* Tbp2, which also has β -sheet regions. These results add to the evidence that the Tbp2 proteins have different structures.

IV. Distribution of *tbpA* in *P. haemolytica* and related species

Southern hybridization analysis was carried out to determine whether or not all sixteen serotypes of *P. haemolytica* carried the gene for the Tbp1 protein. Chromosomal DNA from each of the serotypes was digested with restriction endonuclease and probed with the 5' end of the *typA* gene from *P. haemolytica* A1. Similar hybridization experiments were performed on digested chromosomal DNA from *A. pleuropneumoniae* CM% and Shope 4074 and *A. suis* 3714.

High-stringency Southern hybridization (50% formamide) with the *P. haemolytica* A1 *tbpA* probe demonstrated the presence of *tbpA* homologous sequences in all sixteen serotypes of *P. haemolytica* (Figures 5,6). In addition, there is a considerable difference in the size of fragments which hybridized with the probe between the A and T biotypes. It is important to note that in Figure 5, there was a problem with the quality of the serotype 7 DNA, which did not give a reactive band with Southern hybridization. The reaction of this serotype should be identical to that of serotype 1. A similar problem is seen in Figure 6, where serotypes 12 and 16 DNA was not properly digested. The reaction of these serotypes should be identical to that of serotype 1.

Low-stringency Southern hybridization (25% formamide) with the *tbpA* probe indicated that *A. suis* 3714, *A. pleuropneumoniae* CM5 and Shope 4074 genomic DNA hybridized with the *P. haemolytica* *tbpA* probe (Figure 7). The two strains of *A. pleuropneumoniae* both belong to serotype 1 and hybridized in the same fashion. A preliminary restriction map of the *tbpA*, *tbpB* regions in *P. haemolytica* A1, *A. suis* and *A. pleuropneumoniae* is shown in Figure 8.

V. Homology Studies

The predicted amino acid sequence of *P. haemolytica* Tbp1 was compared with the predicted sequences for the *Neisseria* spp. and *A. pleuropneumoniae* transferrin binding proteins as well as for several *E. coli* TonB-dependent receptor proteins. All of the comparisons were performed according to the Higgins and Sharp algorithm (Higgins and Sharp, 1988).

The predicted amino acid sequence of *P. haemolytica* Tbp1 was found to have a high degree of homology with both the *N. gonorrhoeae* and *N. meningitidis* Tbp1 proteins (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993) (Figure 9). The homology, including identical and conserved amino acids, was found to be 41%. This result agrees with the protein topology studies which suggested that the *P. haemolytica* and *Neisseria* spp. Tbp1 proteins share a similar structure. A homology comparison between *P. haemolytica* Tbp1 and *A. pleuropneumoniae* serotype 7 and serotype 1 TfbA proteins (Gerlach *et al.*, 1992a; Gerlach *et al.*, 1992b) reveals only a low degree (22%) of homology (Figure 10). The degree of genetic relatedness among the *Pasteurella*, *Neisseria*, and *Actinobacillus* transferrin binding proteins is shown in the form of a dendrogram in Figure 11. It is interesting to note that *P. haemolytica* Tbp1 is more closely related to *Neisseria* Tbp1 than to *Actinobacillus* transferrin binding proteins.

P. haemolytica Tbp1 also has localized regions of homology with *E. coli* TonB dependent outer membrane receptors (Figure 12). Homology with these proteins implies the *P. haemolytica* Tbp1 is also a TonB dependent receptor protein. The first homologous domain includes the TonB box, which has been implicated in the direct interaction between TonB and the receptor protein (Bell *et al.*, 1990). The significance of the other homologous domains is not known, however, it is possible that they are also involved in TonB interaction.

VI. T7 expression of Tbp1

T7 expression was performed in order to express the protein encoded by *tbpA* (Figure 13). An attempt to express *tbpA* by maxi-cell analysis of *E. coli* under iron-depleted and iron-repleted conditions was not successful (data not shown). The T7 expression did not produce any reactive band at 100 kDa. A 30 kDa positive control is shown in lane 1. There was no difference between the plasmid carrying *tbpA* and the pBluescript vector alone (Figure 13, lanes 2 and 3).

VII. Western Immunoblot Analysis

Inner and outer membrane fractions from *P. haemolytica* A1 and *E. coli* HB101 cells grown under iron-limiting and iron-sufficient conditions, were prepared and analyzed by Western immunoblotting. The purpose of these experiments was to determine whether or not the iron-regulated proteins would react antigenically with antiserum prepared against the soluble antigens of *P. haemolytica*. The inner and outer membrane fractions were immunoblotted with rabbit "anti-autologous" antiserum which was raised against the soluble antigens of *P. haemolytica* A1 cultured in RPMI 1640 that had been supplemented with the rabbit's own serum (to avoid inclusion of antibodies to serum proteins). The antiserum was preabsorbed with *E. coli* HB101 cells in order to minimize reactivity with *E. coli* antigens. Immunoreactive bands

corresponding to the transferrin binding proteins were not observed in the outer membrane fraction from *P. haemolytica* A1 cells grown under iron-limiting conditions (Figure 14, lane 3).

The inner and outer membrane fractions were also immunoblotted with the serum from a calf vaccinated with Presponse® as a first antibody (Figure 15). The antiserum was preabsorbed with *E. coli* HB101 cells to limit the number of *E. coli* immunoreactive. Bands of 71, 77 and 100 kDa were observed in the outer membranes of *P. haemolytica* cells which were grown under iron-limiting conditions (Figure 15, lane 3). These protein bands correspond to the size of the *P. haemolytica* transferrin binding proteins. If these antigenic bands are the transferrin-binding proteins then this result suggests that these peptides are antigenic and also immunogenic in cattle.

DISCUSSION

I. Preliminary Sequence analysis

The preliminary nucleotide sequences of *P. haemolytica* *tbpA* and *tbpB* are shown in Figure 3. The promoter region of *P. haemolytica* *tbpB* is shown in Figure 4.

Upon preliminary sequence analysis of the cloned DNA, the two *tbp* genes were found in tandem with *tbpB* directly upstream of *tbpA*. This genetic organization is consistent with iron uptake systems in other bacteria such as *Neisseria* spp. where the genes where are arranged in an operon (Anderson *et al.*, 1994). It is likely that the genes involved in *P. haemolytica* A1 iron uptake are also arranged in an operon. The *tbpA* gene has only a ribosomal binding sequence whereas the *tbpB* gene is preceded by a ribosomal binding site and has a Fur consensus sequence in its promoter region.

The presence of a putative Fur consensus sequence implies that the two genes could be coordinately regulated by iron concentrations and that a Fur homolog exists in *P. haemolytica* A1. Fur homologs have been cloned and sequenced in pathogenic *Neisseria* Spp. (Berish *et al.*, 1993; Thomas and Sparling, 1994). If a *P. haemolytica* A1 Fur homolog exists, it may be involved in the regulation of other antigens such as the leukotoxin. Strathdee and Lo (1989) reported that under iron-limiting conditions, there was a decrease in the amount of leukotoxin produced. This is the opposite of the situation in the diphtheria toxin (Boyd *et al.*, 1990) where toxin production increases when the cells are grown under iron-limiting conditions. It is possible that Fur acts as a positive regulator in *P. haemolytica* leukotoxin production. This would be consistent with the earlier observation by Gentry *et al.* (1986) of increased toxin production in iron containing media. *N. meningitidis* also produces iron-regulated proteins which are related to the RTX family of exoproteins (Thompson *et al.*, 1993).

The first 28 predicted amino acids in the *tbpA* sequence form a putative signal sequence. A signal sequence is essential for inserting the precursor protein into the membrane during the process of translocation across the membrane. The signal sequence also acts as a recognition site for the proteolytic cleavage of the precursor protein into its mature form (von Heijne, 1983; Benson and Silhavy, 1983). The presence of a signal sequence confirms that Tbp1 is

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located beyond the cytoplasmic membrane, but it does not contain any sorting information. The predicted amino acid sequence of Tbp1 has a terminal phenylalanine residue at the carboxyl-terminal of the protein. Phenylalanine is a hydrophobic aromatic amino acid which facilitates the partitioning of the hydrophobic environment in the membrane. The presence of a terminal
5 phenylalanine residue has been shown to be important for outer membrane localization (Struve *et al.*, 1991) and suggests that *P. haemolytica* Tbp1 is located in the outer membrane.

Sequence analysis of *tbpB* revealed a putative cleavage sequence for lipoproteins. Lipoproteins have a characteristic cleavage sequence of Leu-X-Y-Cys, where X and Y are small neutral amino acids (Wu, 1987). This suggests that Tbp2 is processed and lipid modified. It is
10 interesting to note that Tbp2 lacks a terminal phenylalanine residue which is involved in outer membrane localization. Analogous transferrin binding lipoproteins have been found in *H. influenzae*, *N. gonorrhoeae* and *N. meningitidis* (Legain *et al.*, 1993; Anderson *et al.*, 1994) Griffiths *et al.* (1993) has demonstrated common antigenic domains among the Tbp2 proteins in *N. gonorrhoeae*, *N. meningitidis* and *H. influenzae* type b.

15 The isoelectric point (pI) of a protein is defined as the pH at which the peptide has a net charge of zero. The pI calculation assumes that there are no three-dimensional structures which interfere with the ionization states. Therefore, calculated pI values are only approximate values and may differ from experimental results. The pI of *P. haemolytica* Tbp1 and Tbp2 has been calculated to be 9.16 and 9.71, respectively. It has been suggested that
20 cationic polypeptides enhance *in vivo* membrane interactions. It is possible that the basic nature of Tbp1 enhances interaction with the host transferrin proteins. The *Neisserial* Tbp1 (Cornelissen *et al.*, 1992) and Fbp (Berish *et al.*, 1990) proteins are also basic proteins. In *Legionella pneumophila*, basic surface proteins act to inhibit phagolysosomal fusion (Cianociotto *et al.*, 1989).

25 II. Preliminary Predicted protein topology

The hydropathy plots of Tbp1 and Tbp2 were generated using the method of Kyte and Doolittle (1982). In the hydropathy plot of *P. haemolytica* Tbp1, the first peak is located from the amino acids 1 to 30. This represents the hydrophobic core which is common to all signal
30 sequences (Hayashi and Wu, 1990). The other hydrophobic regions may be transmembrane domains. Hydrophilic domains may be regions of the protein which are exposed to either the cell surface or to the periplasm. The location of the transmembrane regions in the *P. haemolytica* Tbp1 protein is similar to many transmembrane regions predicted in *N. gonorrhoeae* Tbp1. This suggests that the Tbp1 proteins may have a similar structure and that they share a certain degree of homology at the amino acid level. The *P. haemolytica* Tbp2 protein possesses
35 a hydrophobic leader sequence as well as several large hydrophobic regions in the centre of the protein and it is significantly different than the hydropathy plot predicted for *N. gonorrhoeae* Tbp2. This implies that both proteins are structurally different.

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Surface exposed regions of Tbp1 and Tbp2 were predicted using the Emini surface probability method. Regions of Tbp1 and Tbp2 which are exposed to the cell surface may be involved in ligand interaction and may be antigenic.

The Chou-Fasman method is commonly used in predicting the secondary structure of a protein. This method is based on the tendency each amino acid has for being in an α -helix, a β -sheet or a β -turn. The Chou-Fasman method predicts that *P. haemolytica* Tbp1 consists of many β -sheets and β -turns. It is possible that these β -sheets cross the outer membrane repeatedly and that the intervening sequences constitute surface or periplasm exposed loops. The Chou-Fasman method also predicts that *N. gonorrhoeae* Tbp1 also consists of β -sheets. This structure has already been proposed for *E. coli* outer membrane proteins such as FepA (Moeck *et al.*, 1994). *P. haemolytica* and *N. gonorrhoeae* Tbp1 proteins share a common structure which implies that they may also have a similar mechanism of removing iron from the host transferrin molecule. The Chou-Fasman plot of *P. haemolytica* Tbp2 also predicts a predominantly β -sheet and β -turn structure which is significantly different than the prediction for *N. gonorrhoeae* Tbp2.

III. Distribution of *tbpA* in *P. haemolytica* and Related Species

Southern hybridization of the genomic DNA of the sixteen *P. haemolytica* serotypes with the *tbpA* probe demonstrated that a highly homologous gene is present within the A biotype. The results also suggest that the genetic organization of the *tbpA* gene is significantly different in the T biotypes. This supports the observations by Murray *et al.* (1992) who demonstrated that iron-regulated proteins from the A and T biotypes were antigenically distinct. Previous work on *P. haemolytica* antigenic determinants demonstrated that the sialoglycoprotease, the serotype specific antigen, three lipoproteins and a LPS biosynthetic gene were either missing or had a different genetic organization in the T biotype (Burrows, PhD thesis, 1993). The A and T biotypes do share phenotypic and biochemical traits (Holt, 1977), but they are only distantly related based on DNA:DNA hybridization (Bingham *et al.*, 1990). Sneath and Stevens (1990) proposed that the biotype T serotypes be renamed as the species *P. trehalosi*.

Diversity in the genetic organization of transferrin binding proteins has also been demonstrated in *A. pleuropneumoniae* TfbA (Gonzalez *et al.*, 1990; Gerlach *et al.*, 1992b) and *N. meningitidis* Tbp2 (Legrain *et al.*, 1993; Rokbi, 1993). In *A. pleuropneumoniae*, serotype 1 and serotype 7 TfbA proteins share only 55% homology at the amino acid level (Gerlach *et al.*, 1992b). *N. meningitidis* Tbp2 proteins are divided into two classes based on their molecular weight, sequence similarity and antigenic heterogeneity (Robki *et al.*, 1993). The diversity in transferrin binding proteins within a species may facilitate the binding of different serotypes to avoid the host immune response against heterologous strains (Gerlach *et al.*, 1992b).

Southern hybridization experiments demonstrated that chromosomal DNA from *A. pleuropneumoniae* strains CM5 and Shope 4074 hybridized with the *tbpA* probe only under low-stringency conditions. This suggests that *P. haemolytica* and *A. pleuropneumoniae* transferrin

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binding proteins share only a low degree of homology. This result was confirmed by homology studies on the amino acid sequence from both *P. haemolytica* Tbp1 and *A. pleuropneumoniae* TfbA proteins.

The *A. suis* genomic DNA also hybridized with the *tbpA* probe, which suggests that it may have an analogous transferrin binding protein. The results also suggest that *A. suis* transferrin binding proteins may be more closely related to the Tbp1 and *P. haemolytica* than to *A. pleuropneumoniae* TfbA.

IV. Homology Studies

All of the protein sequences were aligned by PCGene (Clustal), which compares sequences according to the method of Higgins and Sharp (1988). The first step in this method is to calculate all pairwise sequences similarities. A dendrogram is then generated from the similarity matrix generated in the first step. The dendrogram in Figure 11 was generated by a Higgins and Sharp alignment of the *Pasteurella*, *Actinobacillus*, and *Neisseria* transferrin binding proteins.

a) *Neisseria* spp.

The predicted amino acid sequence of *P. haemolytica* *tbpA* has regions of homology with the predicted amino acid sequence of *tbpA* in *N. gonorrhoeae*. This suggests that the transferrin binding proteins are structurally similar and agrees with the observations made in the protein topology studies. Ogunnariwo and Schryvers (1990) reported that *P. haemolytica* A1 Tbp1 was similar to *N. gonorrhoeae* Tbp1 proteins in size and properties. Both species produce 100 kDa receptor proteins which cannot bind transferrin after SDS-PAGE, which suggests that the conformation of the native protein is important in transferrin binding. However, the two proteins differ in their binding specificities: *N. gonorrhoeae* Tbp1 bound only human transferrin whereas *P. haemolytica* A1 Tbp1 bound only bovine transferrin. This suggests that differences between the two *tbpA* sequences may be regions which encode for specificity of iron source.

b) *A. pleuropneumoniae*

The predicted amino acid sequence of *P. haemolytica* A1 Tbp1 has a low degree of homology with the sequence of *A. pleuropneumoniae* TfbA. This result is confirmed by the Southern hybridization experiments, which demonstrated that chromosomal DNA from *A. pleuropneumoniae* strains CM% and Shope 4074 hybridized with the *tbpA* probe only under low-stringency conditions. This is interesting because both bacteria belong to the family *Pasteurellaceae* and would therefore be expected to have a similar transferrin binding protein. Previous work has suggested that the two proteins are functionally similar but structurally different. The TfbA protein in *A. pleuropneumoniae* has been shown to be a lipoprotein (Gonzalez *et al.*, 1990), whereas the 100 kDa *P. haemolytica* A1 Tbp1 is not (Ogunnariwo and Schryvers, 1990). *A. pleuropneumoniae* is able to distinguish between iron-saturated and iron-

depleted transferrin) (Gerlach *et al.*, 1992a), whereas *N. meningitidis* cannot (Tsai *et al.*, 1988). It is interesting to note that *A. pleuropneumoniae* TfbA has homology with *N. gonorrhoeae* Tbp2, which is also a lipoprotein. This suggests that the TfbA protein is analogous to Tbp2, and that Tbp1 of *A. pleuropneumoniae* has not yet been identified.

5 c) TonB dependent-receptor proteins

The *P. haemolytica* Tbp1 sequence also has amino acids which are common to a group of *E. coli* TonB dependent receptor proteins. This finding suggests that *P. haemolytica* A1 belongs to this family and that a TonB homolog exists in *Pasteurella* species. The first homologous domain or "TonB box" has been implicated in direct interaction between the receptor protein and TonB (Bell *et al.*, 1990, Brewer *et al.*, 1990). The significance of the other homologous regions is not known, but they may be required for TonB interaction or may be necessary for outer membrane localization. *P. haemolytica* Tbp1, like many other TonB-dependent proteins, is a transmembrane protein which is iron-regulated and involved in iron utilization (Mietzner and Morse, 1994). It is possible that *P. haemolytica* Tbp1 functions as a gated channel as has been proposed for *E. coli* FepA (Rutz *et al.*, 1992). Tbp1 from both *N. gonorrhoeae* (Cornelissen *et al.*, 1992), and *H. influenzae* (Jarosik *et al.*, 1994) also belong to the family of TonB-dependent receptor proteins.

V. Proposed Model for *P. haemolytica* Iron Uptake

The existence of many analogous proteins in *Neisseria*, *Pasteurella* and *Haemophilus* suggests that a common mechanism may be utilized for iron acquisition. A hypothetical model of iron acquisition has been proposed for *Neisseria* (Chen *et al.*, 1993) which may be used as a model for *P. haemolytica* A1. Iron deprivation activates transcription of the iron-regulated proteins by a Fur-like regulatory system. Host transferrin binds to the bacterial cell surface via a specific iron receptor complex composed of two or more proteins. The iron is removed from the transferrin and transported across the outer membrane of the bacterium with the energy provided by TonB. In the periplasm, the iron is transiently complexed to a periplasmic component, Fbp, which transports it to a cytoplasmic membrane permease. The iron is transported across the cytoplasmic membrane by a periplasmic binding protein transport system. In the cytoplasm the iron is reduced to Fe²⁺ and assimilated by the cell.

One feature which may be unique to *P. haemolytica* A1 iron uptake is the presence of a third iron-regulated outer membrane protein (71 kDa) which may form part of the receptor complex (Ogunnariwo and Schryvers, 1990). In addition, *P. hemolytica* does not have a receptor protein which is capable of binding transferrin after SDS-PAGE and electroblotting, while *N. gonorrhoea* does (Schryvers and Morris, 1988). This suggests that the binding mechanism of *P. haemolytica* receptor complex may be slightly different than the receptor complex in *N. gonorrhoea*.

Proteins which are similar to *N. gonorrhoea* Fbp have been identified in the family Pasteurellaceae. In *H. influenzae*, a 40 kDa periplasmic protein was identified and its N-

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terminal sequence was found to be 81% homologous to *N. gonorrhoea* Fbp (Harkness et al., 1992). In *P. haemolytica* A3, 35 kDa periplasmic iron-regulated protein has been described but no function has been found (Lainson et al., 1990). In addition, a 37 kDa iron regulated protein has been isolated by affinity procedure from *P. haemolytica* A1 (Ogunnariwo and Schryvers, 1990).

- 5 Based on size and location similarities, it is possible that both of these proteins are analogous to *N. gonorrhoea* Fbp.

VI. T7 Protein Expression

- T7 RNA polymerase-dependent production of a Tbp1 gene product in *E.coli* JM109 (DE3) was not successful (Figure 13). One possible explanation may be that the ribosomal
10 binding site of *tbpA* was inefficient. The gene could perhaps be cloned into a vector that carries a functional ribosome-binding site. Alternatively, the Tbp1 protein may be unstable and requires the presence of other proteins or factors in order to be correctly produced. Components of heterodimeric proteins are often unstable when they are synthesized singly.

VII. Western Immunoblot Analysis

- 15 Western immunoblots were performed on the inner and outer membrane fractions from *P. haemolytica* A1 cells which were grown under iron-sufficient or iron-limiting conditions (Figures 14 and 15). The iron-limiting conditions were simulated by adding the iron chelator EDDA, which is a common synthetic iron chelator used to limit the availability of iron in culture media. EDDA was chosen for these studies because of its specificity for iron and its lack
20 of toxic side effects to bacteria (Neilands, 1981).

- P. haemolytica* A1 membrane fractions immunostained with rabbit antiserum to soluble antigens did not react with the iron-regulated proteins (Figure 14). Neither the 100 kDa nor the 77 kDa iron-regulated proteins were observe in this immunoblot, possibly because the original *P. haemolytica* A1 culture (used in the hyperimmunization of the rabbit) was not grown
25 under iron-restricted conditions. The medium used contained 7% serum to avoid the inclusion of antibodies of serum proteins. In contrast, peptides which may be the iron-regulated proteins reacted with antisea from calves vaccinated with the Presponse® (Figure 15). Presponse® is produced from *P. haemolytica* A 1 cells which are grown to late log phase in serum-free RPMI medium 1640 (Shewen and Wilkie, 1987; Shewen et al., 1988). It is possible that the low iron
30 concentration of this medium induced production of the transferrin binding proteins. It is also possible that the calf responded to transferrin binding proteins produced by *P. haemolytica* which are commensal organisms in the nasopharynx. The presence of antibodies to transferrin binding proteins suggests that these proteins are immunogenic.

EXAMPLE 2

- 35 **Bacterial strains.** The bacterial strains used in this study are listed in Table 2. *P. haemolytica* strains h173, h174, h175 and h176 were field isolates from ruminants with pneumonic pasteurellosis and were provided by Dr. Frank Milward, Rhone Merieux, Lyon, France. *P. haemolytica* strains h44-h46 were bovine clinical type A1 isolates from bovine pneumonia

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obtained from S. Lundberg ,Veterinary Laboratory, Regional Agricultural Building, Airdrie, Alberta. h44 has been described previously (26). *P. haemolytica* strains h93-h97 were bovine clinical type A1 isolates from bovine pneumonia obtained from by Dr. A. Potter of the Veterinary and Infectious Diseases Organization (VIDO), Saskatoon. Strains h98-h107 are 5 ATCC *P. haemolytica* strains (5) also obtained from Dr. A. Potter. *Actinobacillus* (*Haemophilus*) *equuli* strain h50 was obtained from Dr. Jane Pritchard, Veterinary Laboratory, Regional Agricultural Building, Airdrie, Alberta. new species, *P. trehalosi* (34).

Table 2. List of strains included in this study.

Species	Strain	Serotype	Host Species	Source
<i>P. haemolytica</i>	h44	A1	cattle	S. Lunberg, Airdrie
<i>P. haemolytica</i>	h45	A1	cattle	S. Lunberg, Airdrie
<i>P. haemolytica</i>	h46	A1	cattle	S. Lunberg, Airdrie
<i>P. haemolytica</i>	h93 (ph21)	A1	cattle	A. Potter, VIDO
<i>P. haemolytica</i>	h94 (ph24)	A1	cattle	A. Potter, VIDO
<i>P. haemolytica</i>	h95 (ph27)	A1	cattle	A. Potter, VIDO
<i>P. haemolytica</i>	h96 (ph45)	A1	cattle	A. Potter, VIDO
<i>P. haemolytica</i>	h97 (ph46)	A1	cattle	A. Potter, VIDO
<i>P. haemolytica</i>	h196	A1	cattle	R. Lo, U. of Guelph
<i>P. haemolytica</i>	h98 (ATCC33366)	A2	sheep	A. Potter, VIDO
<i>P. haemolytica</i> *	h99 (ATCC33367)	T3	sheep	A. Potter, VIDO
<i>P. haemolytica</i> *	h100 (ATCC33368)	T4	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h101 (ATCC33370)	A6	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h102 (ATCC33371)	A7	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h103 (ATCC33372)	A8	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h104 (ATCC33373)	A9	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h105 (ATCC33369)	A5	sheep	A. Potter, VIDO
<i>P. haemolytica</i> *	h106 (ATCC33374)	T10	sheep	A. Potter, VIDO
<i>P. haemolytica</i>	h107 (ATCC33375)	A11	goat	A. Potter, VIDO
<i>P. haemolytica</i>	h173 (77020-15184)	Untypable	goat	F. Milward, Rhone Merieux
<i>P. haemolytica</i>	h174 (90020-16266)	A7	goat	F. Milward, Rhone Merieux
<i>P. haemolytica</i>	h175 (84020-15786)	A7	sheep	F. Milward, Rhone Merieux
<i>P. haemolytica</i>	h176 (84020-15792)	A9	sheep	F. Milward, Rhone Merieux
<i>A. equuli</i>	h50		horse	J. Pritchard, Airdrie

* T-type strains are now considered as a new species, *P. trehalosi* (34).

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Growth conditions. All bacterial strains were stored frozen at -70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate agar plates and incubated at 37°C in a 5% CO_2 incubator. Iron-restricted growth was achieved by growing the bacteria in Brain Heart Infusion broth (BH1, Difco Laboratories) or O'Reilly Niven broth (25) supplemented with 2 $\mu\text{g}/\text{ml}$ thiamine monophosphate and 3 $\mu\text{g}/\text{ml}$ nicotinamide adenine dinucleotide (NAD) and containing the iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDHA, Sigma) at a final concentration of 100 μM . Growth experiments for use of different transferrins as iron source was performed as previously described (26).

Preparation of transferrins and derivatives. Bovine transferrin was obtained from Sigma. The preparation of equine (horse), ovine (sheep) and caprine (goat) transferrins (2), the iron loading of transferrins to 30% or 100% saturation (22) and conjugation of horse-radish peroxidase (HRP) to transferrin (37) was essentially as described previously. In the preparation of conjugates of bovine, ovine, caprine and equine transferrins (HRP-bTf, HRP-oTf, HRP-cTf and HRP-eTf), the mixture of HRP and transferrin were subjected to gel filtration after chemical conjugation. The fractions demonstrating maximal activity were pooled, dialyzed and aliquots frozen and stored at -70°C .

Solid-phase binding assays. The solid phase binding assay was essentially derived from methods described previously (32). Aliquots of intact cell suspensions or crude total membrane preparations were spotted onto nitrocellulose/cellulose acetate membranes (HA paper, Millipore Corporation, Bedford, MA) and after drying the HA paper was blocked with buffer containing 0.5% skim milk (blocking solution). For the transferrin binding assay, the paper was exposed to blocking solution containing 450 ng/ml of the HRP-conjugated transferrin, washed and developed with HRP substrate mixture essentially as previously described (32). For assessment of binding of anti-receptor antibody by intact cells a similar procedure was utilized except that the first binding solution contained a 1/1,000 dilution of the anti-TbpA and anti-TbpB antisera and, after washing, the membrane was exposed to a second binding solution containing a 1/3,000 dilution of a HRP-conjugated goat anti-rabbit antibody preparation.

Affinity isolation of transferrin binding proteins (TbpA and TbpB). Bovine, ovine, caprine and equine transferrins were individually coupled to CNBr-activated Sepharose 4B according to the manufacturers instructions using solutions containing 3.5 mg/ml of iron-saturated transferrin. Activated groups were blocked by addition of ethanolamine. Noncoupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride and after further washing the bound transferrin was reloaded with iron using a solution containing 5 $\mu\text{g}/\text{ml}$ FeCl_3 in 0.1 M sodium citrate/0.1 M NaHCO_3 pH 8.6 buffer.

Iron-deficient total membrane (200 mg protein) from *P. haemolytica* or *A. equuli* prepared as previously described (32) was diluted to 2 mg/ml in 50 mM Tris pH 8.0 containing 1.0

M NaCl. The diluted membrane was solubilized by addition of EDTA and sarkosyl to a final concentration of 10 mM and 0.75%, respectively followed by incubation of the mixture at room temperature for 15-30 min with gentle rocking. The solution was centrifuged at 10,000 rpm for 10 min to remove insoluble debris. The supernatant containing the solubilized membrane was
5 applied to a 1.5 x 10 cm transferrin-affinity column and then washed extensively (at least 10 bed volumes) with 50 mM Tris pH 8.0 containing 1.0 M NaCl, 10 mM EDTA, 0.75% Sarkosyl to remove non-specifically bound protein. In experiments using low salt washing conditions the washing buffer contained 100 mM NaCl in lieu of 1M NaCl. In some instances, additional washing with 2-3 bed volumes of washing buffer containing 0.2 M guanidine hydrochloride was
10 necessary to remove contaminating proteins.

Coelution of both transferrin binding proteins (TbpA and TbpB) was achieved by application of 2-3 bed volumes of 2.0 M guanidine hydrochloride in 50 mM Tris pH 8.0, containing 1.0 M NaCl, 1 mM EDTA, 0.01% sarkosyl. The eluant was collected for immediate dialysis against 50 mM Tris pH 8.0. Further treatment with higher concentrations of guanidine
15 hydrochloride usually did not result in any further yield of receptor protein. Individual isolation of TbpA and TbpB was attained by sequential elution with 2 bed-volumes of each buffer containing 0.2, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 guanidine hydrochloride, respectively. The eluates were dialyzed against 3 changes of 3 litres 50 mM Tris pH 8.0 over an 18-hour period and concentrated by ultrafiltration. After SDS-PAGE analysis the fractions from the 0.5 and 0.75 M
20 guanidine HCl elution buffers were pooled for a preparation of TbpB and fractions from the 1.5 and 2 M guanidine HCl elution buffers were pooled for a preparation of TbpA.

Analytical methods. Protein samples were analyzed by SDS-PAGE followed by silver staining as previously described (32). For Western blot analysis, about 1-2 .m of purified receptor proteins or 40 .m of outer membrane protein from iron-poor cells were separated on 10% polyacrylamide
25 gels. Proteins were electrophoretically transferred to nitrocellulose (Millipore, Bedford, MA) overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1% SDS. The filters were blocked with 0.5% skim milk in 20 mM Tris pH 7.5, 500 mM NaCl (TBS) for 30 minutes at room temperature. A 1/300 dilution of the appropriate antibody in the blocking solution was applied to the paper for 1 hour at room temperature followed by two, 10-minute
30 washes each with TBS. A 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horse-radish peroxidase conjugate from BioRad) was allowed to bind for 1 hour at room temperature. The conjugate was removed by three, 10-minute washes in TBS and developed using the HRP-substrate mixture.

Comparison of receptor specificity:

35 Prior studies had demonstrated differences in specificities towards different ruminant transferrins (i.e. cattle, sheep and goat) by transferrin receptors from various pathogenic bacterial species of ruminants (38). This probably reflects differences in the regions of the receptor proteins involved in ligand binding and thus suggests that these regions could not

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serve as the basis of a broad-spectrum transferrin receptor-based vaccine for ruminant pathogens. However, it does not preclude the possibility that a group of related ruminant pathogens, such as the various *Pasteurella* species, may have common ligand binding domains that could provide the basis for generation of a cross-protective response. Thus it was important to determine whether the transferrin receptors from a collection of representative *Pasteurella* isolates possessed the same specificity for ruminant pathogens.

As a preliminary analysis of receptor specificity, a collection of representative isolates were assessed for their ability to utilize various ruminant transferrins as a source of iron for growth (Table 2). A simple plate assay described in the methods section was utilized. The growth of all the representative ruminant isolates of *Pasteurella haemolytica* and *P. trehalosi* was stimulated by Fe-saturated transferrins from ruminant (bovine, caprine and ovine) but not from non-ruminant (equine) hosts. The stimulation of the growth of the equine pathogen, *Actinobacillus equuli* (strain h50), by equine transferrin indicated that the inability of the *P. haemolytica* strains to use equine transferrin as iron source was not due to deficiencies in the preparation.

Table 3: Growth on different transferrins.

Species	Strain	Serotype	Host	Source of Tf for growth			
				bTf	oTf	cTf	eTf
<i>P. haemolytica</i>	h44	A1	cattle	+	+	+	-
<i>P. haemolytica</i>	h173	Untypable	goat	+	+	+	-
<i>P. haemolytica</i>	h174	A7	goat	+	+	+	-
<i>P. haemolytica</i>	h175	A7	sheep	+	+	+	-
<i>P. haemolytica</i>	h176	A9	sheep	+	+	+	-
<i>P. haemolytica</i>	h106	T10	sheep	+	+	+	-
<i>A. equuli</i>	h50		horse	-	-	-	+

As a further assessment of the receptor specificity, binding of transferrin by intact cells or isolated membranes was assessed by a simple binding assay utilizing horse-radish peroxidase (HRP) conjugates of transferrin. Conjugates were prepared from bovine, ovine and caprine transferrin and then tested for their ability to bind to total membranes isolated from iron-deficient cells of several representative strains of *P. haemolytica* and *P. trehalosi*. The results illustrate that all the selected strains were capable of binding the three ruminant transferrins (bovine, caprine and ovine) but not equine transferrin (Figure 16), which is consistent with the results of the growth studies (Table 3). To confirm that the observed binding by all three ruminant transferrins was due to the same receptor in the selected species, competitive binding assays were performed in which the ability of unlabelled ruminant transferrins were tested for their ability to block binding of the labelled transferrins. In these experiments

reciprocal inhibition by the various ruminant transferrins was equally effective, indicating that they bound to the same receptor with similar affinities (data not shown).

The results of the growth and binding studies suggested that bovine, ovine and caprine transferrins were capable of interacting with the receptor components involved in iron acquisition in *P. haemolytica*. The affinity procedures described in the methods section were used to identify the proteins interacting with the ruminant transferrins by employing bovine, caprine or ovine transferrin-sepharose resins. As illustrated in Figure 17, a predominant receptor protein of approximately 100,000 molecular weight was isolated with membrane preparations from the bovine isolate (h44), the caprine isolate (h173) or the ovine isolate (h175) when either bovine (lanes A and B), ovine (lane C) or caprine (lane D) transferrin affinity columns were used. This protein is analogous to receptor proteins of similar size that are found in other bacterial pathogens (18,27,30,31), which have conventionally been termed transferrin binding protein 1 (Tbp1). An alternate name, TbpA has been recommended (21) to be consistent with existing conventions of nomenclature.

A second protein of approximately 60,000 molecular weight was also evident in the samples isolated by affinity chromatography with the ruminant transferrins (lanes B, C and D) using membranes from the bovine isolate (h44). This protein is comparable to the lower molecular weight receptor protein, transferrin binding protein 2 (Tbp2), isolated from other pathogenic bacterial species (18,27,30,31). For reasons alluded to above, the alternate name, TbpB, has been recommended (21). A protein of this molecular weight is also detectable in most samples obtained with the caprine (h173) and ovine (h175) isolates but the presence and yield of this component was sensitive to the conditions of isolation. The characteristically low yield of TbpB (Tbp2) relative to TbpA (Tbp1) observed in these species is not a general property of the bacterial receptor proteins and may even reflect common properties of TbpB from related species.

Neither of the proteins were isolated when equine transferrin-Sepharose was used in the affinity isolation procedure (lane E) indicating that their isolation was specifically due to the presence of ruminant transferrin. When less stringent washing conditions were used during the affinity isolation procedure, additional proteins of approximately 38,000 and 70,000 molecular weight were retained by the affinity column (lane A) when membranes from the bovine (h44), caprine (h173) or ovine (h175) isolate were used. An additional protein of approximately 77,000 molecular weight was also evident in the sample obtained with membranes from the bovine isolate.

Comparison of the immunological properties of the receptor proteins.

The observation that bovine, caprine and ovine transferrins compete for the same receptors suggested that there is conservation at least in the binding domain of the receptors. In order to determine whether there was also a similarity with respect to presence of common immunological epitopes, antisera were prepared against purified receptor proteins from one strain to evaluate their crossreactivity with receptor proteins from other isolates. Affinity

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purified preparations of TbpA and TbpB were obtained from strain h44 (see methods section) and used for generation of monospecific antisera in rabbits. These antisera were then tested against receptor proteins isolated from representative strains of different serotypes including isolates obtained from cattle, sheep and goats. The results in Figure 18, Panel A demonstrate that the anti-TbpB antisera reacted strongly with a protein of approximately 60,000 molecular weight (TbpB) that was affinity isolated with bTf-Sepharose from all of the representative strains. Similarly, the anti-TbpA antisera crossreacted with TbpA isolated from all seven representative strains (Figure 18, Panel B). Extension of this analysis to the additional serotypes of ruminant isolates (Table 2) continued to show considerable cross-reactivity with both receptor proteins (data not shown). These data suggest that both receptor proteins are conserved amongst the different serotypes of *P. haemolytica* causing pneumonic pasteurellosis in cattle, sheep and goats.

Although the immunological cross-reactivity illustrated in Figure 18 indicates that there are conserved epitopes in receptor proteins from different species, there is no indication whether any of these epitopes are exposed at the bacterial surface, where they could serve as effective targets for the host immune effector mechanisms. In order to address this issue, a solid-phase binding assay was used to assess the binding of antireceptor antibodies by intact cells. This assay demonstrated that there was strong binding by cells grown under iron-deficient, but not iron-sufficient conditions, when a selection of bovine type A1 isolates were tested (data not shown). When a selection of sheep isolates of varying serotypes were tested, there was a variable degree of reactivity (Figure 19). Other serotypes of type A *P. haemolytica* strains (h98 and h105, Figure 19) showed considerable reactivity against the anti-TbpA and anti-TbpB antisera. In contrast, the T-type strains (*P. trehalosi*, h99, h100 and h106) showed only very weak reactivity against both of the anti-receptor antisera. However, the fact that there was also weak binding by labelled bTf indicates that there was limited production of receptor proteins under the iron-deficient growth conditions used in this experiment. Thus the lack of reactivity of the anti-receptor antisera cannot be attributed to a lack of surface-exposed, cross-reactive epitopes in the receptor proteins from these species.

EXAMPLE 3

30 Cloning of the transferrin receptor genes from a type A1 strain.

The following materials and methods were used in the studies described in the example:

MATERIALS AND METHODS

Bacterial, plasmids, phages and culture conditions. *P. haemolytica* and *E. coli* strains were from the inventors' laboratory collections. The plasmid clone bank of *P. haemolytica* A1 DNA in pBR322 has been described (Lo et al., 1985). The 1 clone bank containing *P. haemolytica* A1 DNA was obtained from G. Weinstock. *P. haemolytica* A1 strain H196 was from the Veterinary Infectious Diseases Organization (VIDO, Saskatoon, Saskatchewan, Canada). All bacterial

strains were stored frozen at -70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate (*P. haemolytica*) or Luria-Bertani plus antibiotic (*E. coli*) agar plates and incubated at 37°C in a 5% CO₂ incubator.

PCR amplification. The primers for PCR were synthesized in an Applied Biosystems Model 390E Synthesizer and purified according to manufacturer's instructions. PCR was carried out in thin-walled 500 µl tube in a Perkin-Elmer Cetus 480 Thermal Cycler using the PCR coreagents and Taq DNA polymerase as recommended. The PCR conditions consist of 95° C for 2 min., followed by 30 cycles of denaturation, annealing and extension at 95° C (1 min.), 52° C (1 min.) and 72° C (2min.) respectively. A negative control which did not contain template DNA was included in each PCR run.

Mapping of *tbp* region in the genome. Genomic DNA from *P. haemolytica* A1 were digested with a number of restriction enzymes, separated by agarose gel electrophoresis, blotted onto nitrocellulose membrane and hybridized with DNA probes specific for the different regions of *tbpA* or *tbpB* as described. The restriction maps were compared with that obtained from the recombinant plasmids as well as the sequenced regions to verify the correct positions of the *tbp* genes.

Preparation of transferrins and derivatives. Bovine transferrin (bTf) was obtained from Sigma. The apo-form of bTf was produced following a procedure described elsewhere (Mazurier and Spik, 1980). Briefly, bTf was dissolved to a concentration of 0.5-1.0 % in 0.1 M Na acetate, 0.1 M Na phosphate, 25 mM EDTA, and adjusted to (pH 5.5) by adding drops of concentrated glacial acetic acid. The solution was equilibrated overnight at 4°C, and the iron removed using an acrylamide gel column equilibrated with the Na acetate/Na phosphate low pH buffer. The low pH buffer was exchanged using a acrylamide gel column equilibrated with 50 mM Tris-HCl (pH 7.5). Finally, the protein was concentrated using Amicon filter. The N- and C-terminal derivatives of bTf were produced as described (Yu and Schryvers, 1994). Briefly, 80 mg of ConA purified bTf were digested with 2 mg proteinase K in 40 ml 0.1 M Tris-HCl (pH 8.2), 25 mM CaCl₂ at room temperature for 20h. To stop the reaction, phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mg/ml. Five ml of concentrated preparation were applied to a Sephadex G-100 column equilibrated with 50 mM Tris-HCl (pH 8.0), the N-lobe and C-lobe fractions were dialyzed against 50 mM Na acetate (pH 6.9), 1mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂ and applied to a ConA-Sepharose column (which binds glycosylated C-lobe but not N-lobe of bTf). Eluants from the column washed with the buffer just described were retained as the N-lobe containing fraction. The C-lobe containing fraction was eluted by using the same buffer containing 0.2 M methyl- α -D-mannopyranoside. Both C-lobe and N-lobe fractions were dialyzed against 50 mM Tris-HCl (pH 8.0), concentrated by ultrafiltration, and frozen as aliquots at -70°C.

Expression of recombinant receptor protein. *E. coli* strains carrying the appropriate recombinant plasmid (DH5 α F/pCRIIPH_{tbpB} for TbpB and DH5 α F/pCRIIPH_{tbpA} for TbpA) were used to

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inoculate 50 ml LB-broth starter cultures containing 0.2% maltose and 150 mg/ml ampicillin. After growth at 37°C for several hours the cultures were used to inoculate 1 liter of the same medium to a starting OD₆₀₀ of 0.05. Once the OD₆₀₀ reached 0.4, glucose was added to 4 mg/ml and grown until OD₆₀₀ reached 0.7-0.8. At that time MgSO₄ to 10 mM and 100 ml of a 10¹⁰ pfu/ml suspension of CE6 1 phage were added. The cell culture was incubated for an additional 2 hrs at 37°C and then harvested by centrifugation. The cell pellet was resuspended in 5 ml of ice cold 50 mM Tris-HCl pH 8.0, 1 M NaCl for affinity isolation, SDS-PAGE, and Western Immunoblot analysis.

Affinity isolation of transferrin binding proteins and analytical methods. Bovine transferrin was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions using solutions containing 3.5 mg/ml of iron-saturated bTf. Activated groups were blocked by addition of ethanolamine. Non-coupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride. After further washing with 50 mM Tris-HCl (pH 8.0) the bound transferrin was reloaded with iron using a solution containing 5 mg/ml FeCl₃ in 0.1 M sodium citrate/0.1 M NaHCO₃ (pH 8.6). After washing again with 50 mM Tris-HCl (pH 8.0), the bTf-Sepharose resin was pre-equilibrated with 50 mM Tris-HCl (pH 8.0), 1 M NaCl before it was used in affinity experiments. *E. coli* cells containing recombinant plasmids resuspended in 50 mM Tris-HCl (pH 8.0)/1 M NaCl were solubilized in 20 mM EDTA, 2 % Sarkosyl and incubated for 2 h at room temperature. The mixture was centrifuged for 15 min at 8,000 rpm (4°C) and the supernatant containing the solubilized receptor was carefully decanted. The supernatant was diluted 4 times with 50 mM HCl (pH 8.0), 1 M NaCl buffer and pre-incubated for 30 min at room temperature with an excess (1 mg/ml) of each of the following transferrins diluted in the same buffer: iron-loaded bTf, goat or caprine transferrin (cTf), sheep or ovine transferrin (oTf), and human transferrin (hTf); apo-bTf; C-lobe bTf; and N-lobe bTf. Only the buffer was added to the positive control experiment. After pre-incubation the supernatants were applied to a bTf-Sepharose column previously equilibrated with 50 mM Tris-HCl (pH 8.0), 1 M NaCl and incubated for 15 min at room temperature. Each column was washed extensively with at least 12 column volumes of 50 mM Tris-HCl, (pH 8.0), 1 M NaCl, 10 mM EDTA, 0.5% sarkosyl followed by 10 column volumes containing only 0.05% sarkosyl to remove non-specifically bound protein. Final wash was done with 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl.

Elution of the recombinant TbpB was achieved by application of 1 bed volume of 2 x SDS-PAGE sample buffer under non-reducing condition and no boiling. Each eluant (supernatant) was collected after centrifuging the mixture containing the resin for 5 min at 13,000 x g in a microfuge. An aliquot of each supernatant (eluant) was further submitted to SDS-PAGE and electroblotted to Immobilon PVDF (Millipore) membrane (overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1% SDS). The membrane was blocked with 0.5% skim

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milk in 20 mM Tris (pH 7.5), 500 mM NaCl (TBS) for 30 minutes at room temperature. A 1/1,000 dilution of the anti-TbpB serum in the blocking solution was applied to the membrane for 1 hour at 37°C followed by two, 10-minute washes each with TBS. A 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate) was allowed to bind for 1 hour at 37°C. The conjugate was removed by three, 10-minute washes in TBS and developed using an HRP-substrate mixture (Chloro-Naphtol/H₂O₂).

N-terminal amino acid sequence analysis. Samples of affinity-purified and sequentially eluted TbpA and TbpB from H196 were subjected to SDS-PAGE, electroblotted onto PVDF (Immobilon-P, Millipore IPVH 00010) membrane, briefly stained with Coomassie Blue, and strips containing the individual protein bands were cut from the membrane for N-terminal amino acid sequence analysis.

Preparation of anti-TbpA and anti-TbpB monospecific rabbit sera. Approximately 500 mg of purified TbpA and TbpB from *P. haemolytica* strain H44 obtained from the appropriate fractions in the affinity procedure after dialysis and concentration was mixed with Freund's complete adjuvant and injected intramuscularly into two white female New Zealand rabbits, respectively. The rabbits were boosted twice at 3-week intervals with the same amount of antigens plus Freund's incomplete adjuvant. Two weeks after the final boost, blood was collected to determine the serum titre to the respective antigens using the dot assay in a dot-blot apparatus. The rabbits were either further boosted if titre was unsatisfactory or terminally bled, if the titre was satisfactory. The specificity of the sera against TbpA and TbpB from H44 was examined by SDS-PAGE and Western Immunoblot analysis using goat anti-rabbit IgG conjugated to HRP as secondary antibody. Expectedly, both TbpA and TbpB antisera crossreacted with TbpA and TbpB from strain H196, respectively.

Nucleotide sequence analysis. Two separate strategies for sequencing the *tbp* region were adopted. One approach primarily involved subcloning fragments from recombinant plasmids into the M13 vectors and then sequencing subsequently isolated single stranded DNA prepared by the dideoxy chain termination method using vector primers. In a limited number of cases, oligonucleotide primers were synthesized on the basis of the sequence results from the cloned inserts and used to complete the sequence of the cloned insert. In this analysis the nucleotide sequences were compiled and analyzed by the Pustell programs (IBI).

An alternate approach primarily involved sequence determination of a succession of cloned inserts obtained by PCR amplification from chromosomal DNA. Oligonucleotide primers were synthesized on the basis of the preceding sequence analysis. The PCR amplified products were cloned into the pCRII cloning vector (Invitrogen). Double stranded DNA sequencing was performed using purified recombinant plasmids by the oligonucleotide primer-directed procedure using synthetic oligonucleotides, fluorescent dye-labelled dideoxynucleotide triphosphate terminators, and cycle sequencing with Taq polymerase. Sequence reaction products were analysed on a Applied Biosystems(ABI) model 373A automated fluorescent sequencer. The

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results from successive sequencing runs were compared and the composite sequence was determined by comparison of the chromatograms using the SeqEd program. This sequence was subsequently compared to the sequence obtained by single strand sequencing using the MacDNASIS program. In addition the sequence was analyzed by comparing the predicted protein sequence in all three reading frames with aligned sequences for Tbps from several different species. Any areas of uncertainty identified by this analysis were subjected to repeated runs of sequence analysis.

RESULTS

Cloning the transferrin receptor genes. Anti-receptor antisera and N-terminal amino acid sequences were obtained in order to facilitate cloning of the *P. haemolytica* transferrin receptor genes. Monospecific antisera was obtained by immunizing rabbits with the affinity purified receptor proteins, TbpA and TbpB from a serotype A1 strain (H44) of *P. haemolytica*. Amino acid sequence analysis of an electroblotted preparation of purified native H196 TbpA yielded a readable sequence of 20 amino acids (top of Figure 20). A similar analysis with the purified TbpB failed to provide any sequence information suggesting the N-terminus of this protein may be blocked.

The sequence of the first eight amino acids of the purified TbpA was used to design an oligonucleotide primer based on a *P. haemolytica* preferred codon usage table (tbpA primer 023, Table 4). This primer was used in combination with either of two vector primers (RL2 and RL3, Table 4) for polymerase chain reaction (PCR) amplification of a portion of the *tbpA* gene from a *P. haemolytica* plasmid bank (Lo et al., 1985). An 800 bp PCR product was obtained with vector primers RL2 and 023 and its authenticity was verified by sequence analysis since the predicted amino acid sequence contained a sequence that was identical to the N-terminal amino acid sequence and exhibited homology with other TbpA proteins. The cloned PCR product was used as a hybridization probe for Southern analysis of restriction endonuclease-digested H196 *P. haemolytica* chromosomal DNA and for screening of the plasmid bank. The Southern analysis provided a restriction map of the chromosomal DNA in the *tbp* region for comparison to cloned inserts obtained from the plasmid bank.

Table 4: Oligonucleotide primers.

Primer No.	Description (gene/region - location)	Direction*	Sequence
023	<i>tbpA</i> - 5' end, 1st 8 N-terminal aa's	5'-3'	GGAAGCTTACTGAAAATAAAAAAATC GAAGAA
088	<i>tbpA</i> - 5' end,	3'-5'*	CACTACTTTCCCCAAGCCAG
RL2	pBR322 - upstream of BamH1 site	3'-5'*	GGAATTCCTCCTGTGGATC
198	<i>tbpA</i> - 3' end,	3'-5'*	GCIGCII(G/C)IGCICGIAA(T/C)T(T/A)(T/C)
190	<i>tbpB</i> - 5' end, leader peptide region	5'-3'	CAAAGCTTGCTTG(TC)TCIGGIGG
352	upstream of <i>tbpB</i> - 5' end	5'-3'	AGATCTGGATTCTAAATCAGACCGCTTG TATTTAG

192	<i>tbpB</i> - conserved aa sequence near 3' 5'-3'	GTI(T/A)(A/G/C)IGGIGGITT(C/T)TA(T/C)GG
	end	
401	<i>tbpB</i> - 5' end	5'-3' TAAATTAAAGGAGACATTATGTTTAAACT
350	<i>tbpB</i> - 3' end, flanking NcoI site	3'-5'* CGACGCCCATGGTATTTTTCTATTTGAC
		GTTTTCC
199	<i>tbpB</i> - 3' end, flanking HindIII site	3'-5'* GCGCAAGCTTTTATTTTTCTATTTGACG
349	<i>tbpA</i> - 5' end, BamHI/BglII sites	5'-3' GGATTCAGATCTTAAAGGAGACCCTATC
	upstream of rbs	TAATGATAATG
255	<i>tbpA</i> - 5' end, NdeI site at start codon	5'-3' CCCTATCATATGATAATGAAATATCATC
256	<i>tbpA</i> - 3' end, HindIII site after stop	3'-5'* TAGCGCAAGCTTCTAAAACTTCATTCAAAT

* Direction relative to orientation of coding strand for the relevant gene.

Initially, two strongly hybridizing colonies were identified from the *E. coli* clones. Plasmid p**(clone 9) contained a 9 kb insert which included most of the *tbpA* gene with adjacent downstream regions but was fused with DNA from another chromosomal locus (*fis* in Figure 20). The second plasmid, p**(clone 10), only contained a 1.2 kb insert that was primarily situated around the 5 end of the *tbpA* gene.

The artificial junction in plasmid pRYCL9 was reminiscent of similar artifacts observed while attempting to clone the meningococcal *tbpB* gene and the ensuing difficulties that were encountered (23) prompted consideration of alternative strategies for cloning the *P. haemolytica* *tbpB* region. One strategy was based on the observation that in other species the *tbpB* gene was located upstream of the *tbpA* gene (19, 20, 23) and that there were short stretches of amino acid identity in the predicted sequences of the respective TbpBs. A conserved amino acid sequence near the carboxy terminus of TbpBs was used to design a degenerate oligonucleotide primer (primer 192, Table 4) to obtain the remainder of the *tbpA* gene, the intergenic region and a portion of the 3' end of the *tbpB* gene. This primer was used in combination with a primer based on the sequence from the 5 end of the *tbpA* gene (primer 088, Table 4) to amplify a 700 bp fragment from H196 chromosomal DNA. The sequence from this insert enabled the design of an oligonucleotide primer based on the authentic sequence of the 3' end of the *tbpB* gene (primer 199, Table 4) which was used in combination with degenerate oligonucleotide based on a conserved amino acid sequence present in the leader peptide region of known TbpBs (oligo 190, Table 4) for PCR amplification. The resulting 2.4 kb PCR product obtained when H196 chromosomal DNA was used as a template contained the authentic 3' end of the *tbpB* gene. When this PCR fragment was cloned in the pCRII vector and used in expression experiments utilizing the T7 promoter, an intact recombinant TbpB was produced indicating that the ribosomal binding site and start of the *tbpB* gene was contained within the insert.

A second strategy utilized anchored PCR in which PstI-digested pBluescript plasmid was ligated to Pst I-digested H196 chromosomal DNA and used as template for a PCR reaction utilizing a primer from the 3' end of the *tbpB* gene (oligo 199, Table 4) and the M13 reverse primer from the vector. The resulting 3.5 kb product was subcloned into the pCRII vector,

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producing a plasmid that contained the entire *tbpB* gene and a considerable amount of adjacent upstream regions (ORF and RNase T in Figure 20).

Further subcloning the *tbp* genes into expression vectors involved PCR amplification with oligonucleotide primers complementary to the 5 and 3 ends of the genes and inclusion of appropriate restriction sites. One set of primers for amplifying the *tbpA* gene (primer 349 and 256, Table 4) involved introduction of a BamHI and Bgl II site immediately upstream of the predicted ribosomal binding site (rbs) so that provision of an exogenous promoter should result in expression due to the presence of the native rbs. An alternate 5 primer (255, Table 4) involved the introduction of an Nde I site at the start codon so that cloning into the pT7-7 expression vector, which supplies a properly positioned ribosomal binding site, was possible. Since the expression experiments preceded definitive identification of the start of the *tbpB* gene, the subcloned *tbpB* gene was obtained by PCR amplification with an upstream sequencing primer (primer 352, Table 4) and a primer containing the authentic 3 end flanked by an Nco I site (primer 350, Table 4).

Characterization of the transferrin receptor genes. As illustrated in Figure 20, the *tbp* genes appear to be in an operon arrangement, with the *tbpB* gene located upstream of the *tbpA* gene. Comparative sequence analysis revealed that the *tbpB* gene was preceded by an open reading frame (ORF) encoding a protein whose sequence was highly identical to RNaseT from *E. coli* and *H. influenzae*. This ORF was in turn preceded by another ORF encoding a protein with considerable identity to hypothetical proteins identified in *influenzae* and *Vibrio parahaemolyticus*. Downstream of the *tbpA* gene is an ORF encoding a protein whose sequence was 70% identical to factor-for-inversion stimulation (FIS protein - recombinational enhancer) proteins from *H. influenzae* and *E. coli*. This effectively demarcates the boundaries of the transferrin receptor protein gene operon and indicates that there are no immediately adjacent genes related to this iron acquisition pathway.

There is a 420 bp region between the end of the ORF encoding the RNaseT homologue and the start of the *tbpB* gene with potential ribosomal binding sites, promoter sites and regulatory sites all present within the last 62 base pairs (Figure 20). The remaining 358 bp intervening region could presumably contain transcriptional termination signals for the RNaseT gene and sequences potentially involved in regulation of the *tbp* operon. The putative promoter region contains 5/6 and 6/6 of the consensus bases for the *E. coli* *s70* -35 and -10 promoter regions, respectively.

Previous studies have demonstrated that the expression of the *P. haemolytica* transferrin receptor proteins is regulated by the level of available iron in the medium (26). The identification of a putative Fur box overlapping the -10 site of the *tbpB* promoter (Figure 20) suggests that regulation by iron may be at the transcriptional level via the action of a Fur homologue in *P. haemolytica*. The putative Fur box had 12/19 bases identical to the *E. coli* Fur binding site consensus sequence (Litwin and Calderwood, 1993).

Between the *tbpB* and *tbpA* genes there is a 96 bp intergenic region which contains a putative ribosomal binding site upstream of the *tbpA* gene but no evident promoter. In addition, there is no evident transcriptional terminators in this region. Downstream of the *tbpA* gene stop codon and the stop codon of the gene encoding the FIS homologue is a 98 bp region with
5 no evident transcriptional terminators.

Sequence analysis of the *tbpA* gene region from serotype A1 *P. haemolytica* strain H196 revealed an ORF of 2,790 bp encoding a protein with a predicted molecular mass of 106,921 Da (Figure 22). The putative signal peptidase cleavage site at residue 28 was confirmed by comparison with the known N-terminal amino acid sequence of the mature protein (top of
10 Figure 22). The predicted amino acid sequence of TbpA was compared to the sequences of TbpA from *N. meningitidis* (23), *N. gonorrhoeae* (8), *H. influenzae* (20) and *Actinobacillus pleuropneumoniae* (19). The localization of identical amino acids between these proteins (bold-underlined amino acids, Figure 22) was compared to the proposed topology of these amino acid segments based on the model predicted by Tommassen (28). It is evident that most of the
15 identical amino acids are clustered in regions corresponding to the short transmembrane β -sheets or in the segments of the internal and external loops that are immediately adjacent to the transmembrane sections. It is interesting to note that there are conserved pairs of cysteines in proposed external loops 4, 6 and 7 and a unique cysteine pair in loop 10 of the *P. haemolytica* TbpA. These likely represent disulfide bridges that would provide structural stability to the
20 external loops.

Analysis of the sequence in the *tbpB* gene from serotype A1 *P. haemolytica* strain H196 revealed an ORF of 1,752 bp encoding a protein with a predicted molecular mass of 63,419 Da (Figure 23). This predicted protein sequence of TbpB was compared to the published sequences of TbpBs from *N. meningitidis* (23), *N. gonorrhoeae* (1), *H. influenzae* (20) and
25 *Actinobacillus pleuropneumoniae* (14). This predicted amino acid sequence included a 18 amino acid leader peptide, a signal peptidase II recognition sequence with a cysteine as the predicted N-terminal amino acid of the mature protein. The presence of an N-terminal cysteine, which has been shown to be lipidated in other species (14, 24), may explain the inability to obtain an N-terminal amino acid sequence for this protein and may serve as the primary means of
30 anchoring the protein to the outer membrane. Regions that aligned with the putative binding regions of the *A. pleuropneumoniae* TbpB (TfbA) recently identified by Gerlach et. al. (36) are indicated by a double underline.

It is apparent that there are several regions of homology found throughout the length of the amino acid sequence which include several short stretches of identical amino
35 acids (Figure 24). Upon closer inspection it is apparent that there is some homology between regions in the N-terminal and C-terminal portions of the protein suggesting there may be an underlying bilobed structure to the protein, analogous to what is observed for transferrins. Thus the sequences YKGYW (aa 185-189) and YRGTW (aa 449-453), FTADFANK (aa 237-244) and

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FDVDFV NK (aa 480-487), GNRFSG (aa 276-281) and GNGFGG (aa 513-518), and LEGGFFG (aa 300-306) and FEGGFY G (aa 546-552) represent consecutive stretches of homologous amino acids in equivalent positions of the N-terminal and C-terminal portions of the protein.

Expression and analysis of the recombinant receptor proteins. The intact *tbpB* and *tbpA* genes

5 were PCR amplified from H196 chromosomal DNA and subcloned into expression vectors for production of recombinant proteins. For initial attempts at expression of the *tbpB* gene, the subcloned *tbpB* gene was obtained by PCR amplification with an upstream primer (352, Table 4) and a primer containing the authentic 3' end flanked by an *NcoI* site (350, Table 4). When the PCR amplified fragment was subcloned into the pCRII vector, all five of the resulting clones
10 were in the same orientation; downstream of the T7 promoter and in the opposite direction of the *lac* promoter. Since the *lac* promoter would not be tightly regulated in a high copy number vector, this result suggests that expression of the insert in *E. coli* may be selected against. Once the sequence of this region was available it became apparent that primer 352 was immediately upstream of the *RNaseT* gene and thus expression of either this gene or the *tbpB* gene could have
15 been responsible for the selective pressure. Expression of TbpB from the T7 promoter was accomplished by infection with CE6 λ phage, which encodes the T7 RNA polymerase. Two hours after infection a protein of the anticipated molecular weight for TbpB was evident and this protein reacted with anti-TbpB antiserum after electroblotting.

There was detectable binding of labelled bovine transferrin (bTf) by
20 immobilized intact cells expressing the TbpB protein and this level of binding did not increase significantly with prior sonication of the cells (data not shown). Although this could be interpreted as proper processing and export of the TbpB to the cell surface in the heterologous *E. coli* system, disturbance of the outer membrane integrity by overexpression of a foreign protein antigen is an equally plausible explanation. The preliminary binding studies suggested that a
25 functional TbpB protein was being produced, indicating that further analysis might enable evaluation of the functional properties of this protein and ascertain its contribution to the previously characterized properties of the native receptor complex. Thus, crude membranes were prepared from cells expressing TbpB and used in affinity isolation experiments designed to evaluate its binding characteristics. These experiments indicated that the recombinant TbpB is
30 capable of being affinity isolated by immobilized bTf and this isolation could be inhibited by an excess of bovine, ovine or caprine Tf, demonstrating its ability to effectively bind to all three of these ruminant Tfs. Human transferrin as well as apo-bTf did not inhibit the affinity isolation of recombinant TbpB by immobilized iron-loaded bTf. This assay also revealed that both N-lobe and C-lobe of bTf effectively blocked binding of recombinant TbpB to the immobilized bTf.

35 For expressing the *tbpA* gene, PCR amplification was performed with a set of primers (oligos 349 and 256, Table 4) that maintained the predicted ribosomal binding site (Figure 21). After subcloning the PCR product into the pCRII vector, no recombinant TbpA was expressed after infection with CE6 phage (encoding T7 RNA polymerase). Sequence analysis of

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one of the clones revealed excision of several base pairs that eliminated ribosomal binding site. Other repeated attempts at subcloning the *tbpA* gene from PCR using 255 and 256 primers (see Table 4) into the *NdeI* site of the pT7-7 vector (which provides an optimally positioned ribosomal binding site) were unsuccessful.

5 Discussion

In the initial studies demonstrating the presence of a transferrin receptor in *Pasteurella haemolytica* (26) only a single receptor protein (TbpA) was isolated by an affinity method that yielded two receptor proteins (TbpA and TbpB) in other species (32). Thus the ability to use iron from bovine transferrin for growth (26) and the specific binding of ruminant transferrins (39) was initially presumed to be largely mediated by this receptor protein. In subsequent studies that demonstrated that the binding region of bovine transferrin was localized to the C-lobe (41), two receptor proteins (TbpA and TbpB) were isolated by a modified affinity method, although the yield of TbpA greatly exceeded that of TbpB. Thus it was not possible to conclusively attribute the observed binding characteristics of the receptor to either receptor protein, and particularly not to TbpB.

The cloning of the *tbp* genes and expression of recombinant TbpB has enabled specific evaluation of its binding characteristics. These studies have demonstrated that TbpB has a similar host specificity as the native receptor complex (TbpA and TbpB) as it specifically binds Tfs from several ruminant species. In contrast, unlike the native receptor complex, the recombinant TbpB was able to recognize binding determinants on the N-lobe as well as the C-lobe of bTf, suggesting that in the previous study (Yu and Schryvers, 1994) an interaction between TbpA and TbpB might have interfered in the ability of TbpB to also bind to the N-lobe of bTf.

In competitive binding assays with immobilized membranes (TbpA and TbpB) from *P. haemolytica* there was no evident preference for the iron-loaded or apo form of bTf (30) which is similar to what has been observed in most other bacterial species (Blanton et al., 1990; 32; Tsai et al., 1988; 32, 37), except *Moraxella catarrhalis* (Yu and Schryvers, 1993). In the present study recombinant TbpB clearly showed a strong preference for the iron-loaded form of bTf. This preference of TbpB may have functional relevance in increasing the efficiency of iron acquisition *in vivo*.

30 Example 4

Vaccine Potential of Recombinant Tbp2 and Authentic Tbp1

The transferrin-binding proteins Tbp1 and Tbp2 are attractive targets for a number of reasons:

- a) Since acquisition of iron from transferrin is likely essential for bacterial survival, an antibody response against these antigens should be protective.
- b) The genes coding for Tbp1 and Tbp2 appear to be conserved within various isolates of *P. haemolytica* A1.

This study deals with testing the vaccine potential of recombinant Tbp2 and authentic Tbp1, alone and in combination, in an experimental *P. haemolytica* challenge model.

Methods

The Tbp1 and Tbp2 proteins were affinity purified from *P. haemolytica* and recombinant *E. coli* outer membranes, respectively, by affinity chromatography using standard techniques as described herein. Vaccines were formulated using a proprietary mineral oil-based adjuvant (VSA3) such that the volume of each dose was 2 cc containing the following amount of each antigen: Tbp2 - 45 mg; Tbp1 - 85 mg when used by itself or 100 mg when combined with Tbp2. In addition, a placebo vaccine was prepared containing sterile diluent in place of antigen. Five groups were included in the trial, including one which received a single immunization of Tbp2 ten days before challenge, and groups which received two immunizations with Tbp2, Tbp1 + Tbp2, placebo, or Tbp1. The interval between primary and secondary immunization was three weeks and all vaccinations were carried out at a farm in Southern Saskatchewan. Vaccines were delivered via the subcutaneous route. Approximately ten days prior to challenge, animals were transported to Saskatoon and housed at the VIDO research station. All groups contained ten animals with the exception of the group receiving Tbp1 in which there were six. This group was not in the original proposal and was added in order to determine the protective capacity of Tbp1 by itself. In addition, one calf which received one immunization with the Tbp2 formulation developed clinical signs of disease unrelated to vaccination and was therefore excluded from the trial. The composition of the vaccine groups is summarized in Table 5.

Table 5. Composition of vaccine groups.

Vaccine Group	Antigens	Immunizations	Animals/Group
1	Tbp2	One	9
2	Tbp2	Two	10
3	Tbp1 & Tbp2	Two	10
4	Placebo	Two	10
5	Tbp1	Two	6

Calves were challenged via the aerosol route by first exposing them to a suspension of bovine herpesvirus-1 strain 108 containing approximately 2.5×10^6 PFU/ml followed four days later by an aerosol of *P. haemolytica* containing approximately 5×10^8 CFU/ml. Animals were examined daily by a veterinarian and animal health technician and the following data was recorded: weight, temperature, nasal scores, depression, strength, respiratory distress and sickness. Each of these criteria with the exception of weight and temperature were scored on a scale of 0-4.

The serological response to vaccination was measured using an enzyme-linked immunosorbent assay (ELISA). Serum samples were collected at the time of the first and second immunizations plus on the day of challenge with BHV-1. The titers are presented as the reciprocal of the serum dilution which resulted in an optical density equivalent to the background plus two standard deviations. Responses against Tbp1, Tbp2 and the *P. haemolytica* leukotoxin were measured. The latter was included as a diagnostic test to determine if animals had been naturally exposed to the organism.

Results

- a) Response to Vaccination: None of the animals showed any adverse reaction to vaccination with any of the formulations used. The serological response to vaccination was determined using an ELISA procedure which measured the serum antibody levels to Tbp1, Tbp2 and the *P. haemolytica* leukotoxin. The latter antigen was included in order to ensure that none of the animals had increased titers due to the natural exposure to the bacteria. The titers against each antigen are shown in Table 6 and it can be seen that the titers against leukotoxin were comparable at the time of initial vaccination (Bleed 1), the second vaccination (Bleed 2), and at challenge (Bleed 3). Animals which have a titer below 3,000 were considered to be clean. Interestingly, none of the animals seroconverted to a significant degree to the Tbp1 antigen. Based upon the inventors experience with Tbp1 from other organisms, the expected titers should be low but it was unexpected that no significant increase in antibody levels would be detectable. All groups which received Tbp2 responded well to vaccination and although the group which received Tbp1 + 2 had titers approximately 1/2 of that in the Tbp2 group, this difference is not significant.

Table 6. Serological response to vaccination.

ELISA Antigen	Vaccine Group	Titer - Bleed 1	Titer - Bleed 2	Titer - Bleed 3
Leukotoxin	Tbp2 (1 dose)	1010	1350	2156
	Tbp2	1136	1547	2656
	Tbp1 & 2	1159	1239	1137
	Placebo	1688	1834	2782
	Tbp1	980	1282	1339
Tbp1	Tbp2 (1 dose)	113	364	229
	Tbp2	203	206	296
	Tbp1 & 2	150	388	367
	Placebo	140	130	226
	Tbp1	57	55	139
Tbp2	Tbp2 (1 dose)	408	639	9871
	Tbp2	397	12154	66697
	Tbp1 & 2	119	7838	27515
	Placebo	360	269	378
	Tbp1	359	433433	478

ELISA titers against leukotoxin, Tbp1 and Tbp2 were determined using serum samples taken at the time of the first immunization (Bleed 1), the second immunization (Bleed 2), and the day of challenge with bovine herpesvirus-1 (Bleed 3). The numbers are expressed as the reciprocal of the dilution equaling a negative control plus two standard deviations.

- 5 b) Mortality: The experimental disease model has been calibrated to obtain 60-70% mortality under normal conditions. However, mortality was higher than usual in this trial, probably because of the extremely cold temperatures to which the animals were exposed throughout the time period after challenge. Daily low temperatures were in the -40°C range and all animals were housed outdoors during the trial. The mortality by group is shown in Table 7 and the only
- 10 group which showed significant protection was that which received both Tbp1 and Tbp2. This is compared to 50% mortality for Tbp2 by itself and 100% for Tbp1. A single immunization with Tbp1 was of no benefit.

Table 7. Group mortality observed during the trial.

	<u>Vaccine Group</u>	<u>Mortality (%)</u>
15	Tbp2 (1 dose)	78
	Tbp2	50
	Tbp1 & 2	10
	Placebo	90
	Tbp1	100

- 20 c) Clinical Signs of Disease: The clinical results are summarized by group in Table 8. It should be noted that Table 8 contains the clinical results for all days of the trial, including those prior to *P. haemolytica* infection on day four. Therefore, only the results from days 4 through ten have been used to determine the protective capacity of the vaccine formulations. The high rate of mortality observed during this trial had the effect of reducing the size of each group to the point
- 25 where differences observed in all of the clinical parameters measured are not statistically significant. However, it is clear that the group which received both Tbp1 and Tbp2 showed lower scores in most categories between days 5 and 7. The groups which received two immunizations with Tbp2 alone also showed reduced clinical signs of disease in survivors although they did not do as well as the combination group. The contribution of Tbp1 to
- 30 protection is unclear at present since there did not appear to be any antibody response to this antigen.

d) Postmortem Results: Necropsies were performed on all animals which died during the trial. In all cases, *P. haemolytica* was cultured from the lungs and the pathology observed was consistent with fibrinous pneumonia caused by *P. haemolytica*.

Conclusions

Two vaccinations with a formulation containing *P. haemolytica* Tbp1 and Tbp2 provided significant protection against experimental bovine pneumonic pasteurellosis. The exact contribution which Tbp1 provides to this protection is unclear since there did not appear to be
5 any serological response to the protein. The beneficial effect may have been due to a cell-mediated immune response.

Immunization with two doses of Tbp2 provided some degree of protection and it may be possible to increase this by testing vaccine formulations containing greater quantities of antigen or a different adjuvant. It is likely that the immunological response to Tbp2 provided the bulk
10 of the protection seen with the combination vaccine.

Vaccination with one dose of Tbp2 or two doses of Tbp1 had no beneficial effect after experimental challenge.

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Table 8

Mean Clinical Scores by Group. Animals were challenged on day 0 with BHV-1 and day 4 with *P. haemolytica*.

Group	Day 0	Day 1	Day 2	Day 3	Mean Weight (kg)			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	232.4	231.6	228.1	225.9	222.9	223.1	223.6	225.7	256.0	256.0	256.0
Tbp2	211.4	209.3	205.3	202.4	201.2	191.5	197.3	195.5	198.8	197.7	197.2
Tbp1 & 2	217.8	218.1	216.0	212.9	211.2	208.8	207.5	208.3	207.7	207.8	208.3
Placebo	223.1	221.2	215.7	213.6	209.5	206.2	194.4	177.0	158.0	161.0	158.0
Tbp1	225.3	225.8	219.8	216.7	212.7	208.2	200.0	193.0	191.0	190.0	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Temperature			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	39.06	38.96	39.92	40.21	40.53	40.00	39.40	39.50	39.25	38.85	39.30
Tbp2	39.05	39.26	40.29	40.50	40.44	40.51	40.19	39.67	39.88	39.33	39.10
Tbp1 & 2	39.19	39.36	40.10	40.00	40.21	40.20	39.48	39.49	39.14	39.26	39.48
Placebo	39.03	39.10	40.30	40.41	40.83	40.24	39.96	40.35	39.50	39.10	40.70
Tbp1	39.15	38.78	40.52	40.12	40.47	40.85	40.95	41.60	40.90	41.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Nasal Score			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.11	0.33	0.61	1.17	1.33	1.40	1.25	0.00	0.00	0.00
Tbp2	0.00	0.05	0.30	0.85	1.20	1.45	1.56	1.44	0.83	0.33	0.20
Tbp1 & 2	0.00	0.10	0.45	0.60	1.05	1.70	0.90	1.00	0.70	0.33	0.44
Placebo	0.00	0.15	0.35	0.95	1.10	1.78	1.40	1.50	1.00	0.00	0.00
Tbp1	0.00	0.08	0.33	0.67	0.83	1.08	1.00	0.00	1.00	2.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Depression Score			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.00	0.00	0.11	0.22	0.67	1.60	1.75	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.15	0.00	0.50	0.89	1.67	0.67	1.33	0.40
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.60	1.20	1.10	0.60	0.33	0.56
Placebo	0.00	0.00	0.05	0.15	0.60	1.33	2.20	2.00	1.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.17	0.50	1.83	1.50	2.00	2.00	3.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Strength Score			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.67	1.60	1.75	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.00	0.00	0.40	0.78	1.67	0.50	1.00	0.40
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.30	0.50	1.10	0.60	0.22	0.11
Placebo	0.00	0.00	0.00	0.00	0.00	0.67	1.60	1.50	1.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.00	0.00	1.17	0.50	1.00	2.00	3.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Respiratory Distress Score			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.11	1.40	2.00	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.00	0.00	0.10	0.44	1.22	0.33	0.67	0.00
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.40	0.50	0.11	0.11
Placebo	0.00	0.00	0.00	0.00	0.00	0.67	1.00	1.00	0.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.00	0.00	1.17	0.50	1.00	1.00	3.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Mean Sick Score			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.00	0.56	0.78	0.89	0.89	2.00	2.25	0.00	0.00	0.00
Tbp2	0.00	0.00	0.90	0.90	1.00	1.00	1.00	1.89	0.83	1.17	0.40
Tbp1 & 2	0.00	0.00	0.60	0.50	0.90	1.10	0.90	1.20	0.70	0.33	0.44
Placebo	0.00	0.00	0.70	1.00	1.20	1.80	2.71	2.67	1.00	0.00	1.00
Tbp1	0.00	0.00	0.83	0.50	1.00	2.83	2.75	3.00	2.00	4.00	N/A

Group	Day 0	Day 1	Day 2	Day 3	Cumulative Mortality			Day 7	Day 8	Day 9	Day 10
					Day 4	Day 5	Day 6				
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.78	0.78	0.78	0.78
Tbp2	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.30	0.40	0.40	0.50
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.10
Placebo	0.00	0.00	0.00	0.00	0.00	0.40	0.50	0.80	0.80	0.90	0.90
Tbp1	0.00	0.00	0.00	0.00	0.00	0.33	0.67	0.67	0.83	0.83	1.00

EXAMPLE 5

Comparison of the transferrin receptor from various ruminant serotypes

A collection of *Pasteurella haemolytica* and *P. trehalosi* strains of various serotypes isolated from cattle, sheep and goats were analyzed for binding of ruminant transferrins and utilization of transferrin iron for growth. Some of the goals of the study were to determine the prevalence of transferrin receptors from different host species, to evaluate their specificities for different ruminant transferrins and to determine if there is antigenic relatedness amongst the surface receptors from the different strains causing shipping fever in cattle, pneumonia in sheep and goats and septicemia in lambs.

10 Materials

Bacterial strains. The bacterial strains used in this study are listed in Table 9. Clinical type A1 isolates of *P. haemolytica* (h93-h97)(9) and representative ATCC strains (h98-h107) from bovine pneumonia were provided by Dr. Andrew Potter, VIDO, Saskatoon. *P. trehalosi* strain h174, field isolate from goat with pneumonic pasteurellosis was provided by Dr. Frank Milward, Rhone Merieux, Lyon, France. *P. haemolytica* strain h44, a bovine clinical type A1 isolate from bovine pneumonia, has been described previously (26). Strain h196 was obtained from Dr. Lo, University of Guelph, Ontario, Canada.

Table 9. Bacterial strains, serotypes and sources.

Species	Strain	Serotype	Source
<i>P. haemolytica</i>	h44	A1	cattle
<i>P. haemolytica</i>	h93	A1	cattle
<i>P. haemolytica</i>	h94	A1	cattle
<i>P. haemolytica</i>	h95	A1	cattle
<i>P. haemolytica</i>	h96	A1	cattle
<i>P. haemolytica</i>	h97	A1	cattle
<i>P. haemolytica</i>	h196	A1	cattle
<i>P. haemolytica</i>	h98 (ATCC33366)	A2	sheep
<i>P. trehalosi</i>	h99 (ATCC33367)	T3	sheep
<i>P. trehalosi</i>	h100 (ATCC33368)	T4	sheep
<i>P. haemolytica</i>	h103 (ATCC33372)	A8	sheep
<i>P. haemolytica</i>	h104 (ATCC33373)	A9	sheep
<i>P. haemolytica</i>	h105 (ATCC33369)	A5	sheep
<i>P. trehalosi</i>	h106 (ATCC33374)	T10	sheep
<i>P. haemolytica</i>	h107 (ATCC33375)	A11	goat
<i>P. trehalosi</i>	h174 (90020-16266)	T3	goat

Growth conditions. All bacterial strains were stored frozen at -70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate agar plates and incubated at 37°C in a 5% CO₂ incubator. Iron-restricted growth was achieved by growing the bacteria in Brain Heart Infusion broth (BH1, Difco Laboratories) or O'Reilly-Niven broth (25) supplemented with 3.0 g/ml nicotinamide adenine dinucleotide (NAD) and containing the iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDA, Sigma) at a final concentration of 100 M. Growth experiments for use of different transferrins as an iron source was performed as previously described (26).

Preparation of transferrins and derivatives. Bovine transferrin was obtained from Sigma. The preparation of ovine (sheep) and caprine (goat) transferrins (2), the iron loading of transferrins to 30% or 100% saturation (Herrington et al.1985) and conjugation of horse-radish peroxidase (HRP) to transferrin (37) was essentially as described previously. In the preparation of conjugates of bovine, ovine and caprine transferrins (HRP-bTf, HRP-oTf and HRP-gTf), the mixture of HRP and transferrin were subjected to gel filtration after chemical conjugation. The fractions demonstrating maximal activity were pooled, dialyzed and aliquots frozen and stored at -70°C.

Transferrin binding assay. The solid phase binding assay for transferrin was essentially as described previously (32). After the membrane or concentrated eluates were spotted onto HA paper (Millipore Corporation, Bedford, MA) and blocked in 0.5% skim milk, the paper was exposed to blocking solution containing 450 ng/ml of the HRP-conjugated transferrin. The incubation, washing and development with HRP substrate mixture were performed essentially as previously described (32).

Affinity isolation of transferrin binding proteins . Bovine, ovine and caprine transferrins were individually coupled to CNBr-activated Sepharose 4B according to the manufacturers instructions using solutions containing 3.5 mg/ml of iron-saturated transferrin. Activated groups were blocked by addition of ethanolamine. Non coupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride and after further washing the bound transferrin was reloaded with iron using a solution containing 5 mg/ml FeCl₃ in 0.1 M sodium citrate/0.1 M NaHCO₃ pH 8.6 buffer.

Iron-deficient total membrane (200 mg protein) from *P. haemolytica* or *P.trehalosi* prepared as previously described (32) was diluted to 2 mg/ml in 50 mM Tris pH 8.0 containing 1.0 M NaCl. The diluted membrane was solubilized by addition of EDTA and sarkosyl to a final concentration of 10 mM and 0.75%, respectively followed by incubation of the mixture at room temperature for 15-30 min with gentle rocking. The solution was centrifuged at 10,000 rpm for 10 min to remove insoluble debris. The supernatant containing the solubilized membrane was applied to a 1.5 x 10 cm transferrin-affinity column and then washed extensively (at least 10 bed volumes) with 50 mM Tris pH 8.0 containing 1.0 M NaCl, 10 mM EDTA, 0.75% Sarkosyl to

remove non-specifically bound protein. In experiments using low salt washing conditions the washing buffer contained 100 mM NaCl in lieu of 1M NaCl. In some instances, additional washing with 2-3 bed volumes of washing buffer containing 0.2 M guanidine hydrochloride was necessary to remove contaminating proteins.

- 5 Coelution of both transferrin binding proteins (TbpA and TbpB) was achieved by application of 2-3 bed volumes of 2.0 M guanidine hydrochloride in 50 mM Tris pH 8.0, containing 1.0 M NaCl, 1 mM EDTA, 0.01% sarkosyl. The eluant was collected for immediate dialysis against 50 mM Tris pH 8.0. Further treatment with higher concentrations of guanidine hydrochloride usually did not result in any further yield of receptor protein. Individual
10 isolation of TbpA and TbpB was attained by sequential elution with 2 bed-volumes of each buffer containing 0.2, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 guanidine hydrochloride, respectively. The eluates were dialyzed against 3 changes of 3 litres 50 mM Tris pH 8.0 over an 18-hour period and concentrated by ultrafiltration. After SDS-PAGE analysis the fractions from the 0.5 and 0.75 M guanidine HCl elution buffers were found to contain TbpB only and were thus pooled for a
15 preparation of TbpB and fractions from the 1.5 and 2 M guanidine HCl elution buffers were pooled for a preparation of TbpA.

- Preparation of anti-TbpA and anti-TbpB monospecific rabbit sera.* Approximately 500 µg of purified TbpA or TbpB from *P. haemolytica* strain h44 prepared as described above, was mixed with Freund's complete adjuvant and injected intramuscularly into two white female New
20 Zealand rabbits. The rabbits were boosted twice at 3-week intervals with the same amount of antigens in Freund's incomplete adjuvant and the immune sera collected 2 weeks after the final boost. The specificity of the sera against TbpA and TbpB was tested after SDS-PAGE and immunoblotting of the receptor proteins and using goat anti-rabbit IgG conjugated to HRP as secondary antibody.

- 25 *Analytical methods.* Protein samples were analyzed by SDS-PAGE followed by silver staining as previously described (32). For Western blot analysis, about 1-2 µg of purified receptor proteins or 40 µg of outer membrane protein from iron-limited cells were separated on 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (Millipore, Bedford, MA) overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1%
30 SDS. The filters were blocked with 0.5% skim milk in 20 mM Tris pH 7.5, 500 mM NaCl (TBS) for 30 minutes at room temperature. The membrane was exposed to 1/1000 dilution of the appropriate antibody in blocking solution for 1 hour at room temperature, washed twice with TBS, and then exposed to a 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horse-radish peroxidase conjugate from BioRad). The conjugate was removed, washed three times
35 with TBS and then developed using the HRP-substrate mixture. For the whole cell assay, iron-deficient or iron-sufficient (control) cells were directly spotted onto HA paper. After drying, the HA paper was treated with blocking solution and washed with TBS and then tested for reactivity with anti TbpA or anti TbpB antisera as described above. A control set of spotted

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cells was treated with HRP-bovine transferrin for 1 hour followed by development with HRP-substrate after washing in TBS.

PCR amplification of *tbp* genes and restriction endonuclease digest analysis. Amplification of *tbpA* and *tbpB* from *P. haemolytica* and *P. trehalosi* strains was performed on intact cells by the method of Saris et al.(1990). Amplification of *tbpA* was carried out with oligonucleotides *tbpA* 5' and *tbpA* 3' (#255 and #256, table2). Oligonucleotides #401 and # 199, (Table 10) were used to amplify *tbpB*. Reaction conditions consisted of 30 cycles of 94°C for 1min, 45°C for 1min, and 74°C for 2min. The PCR product was separated by 1% agarose gel electrophoresis in 0.5X TBE buffer (45mM Tris-borate, 1mM EDTA, pH8.3) and stained with 0.5mg of ethidium bromide per ml in the same buffer. For Sau3A restriction endonuclease (Gibco BRL) digestion, PCR products were subjected to phenol chloroform and ethanol precipitation and thereafter were digested with Sau3A. The digests were analysed with a 7.5% acrylamide gel run in 0.5X TBE buffer and visualised in the same way as the agarose gels described above.

Results

Specificity of receptor binding. In a prior study it was demonstrated that transferrin receptors in representative isolates from pathogenic bacterial species varied in their interaction with goat, sheep and cattle transferrin (Yu and Schryvers 1996). Thus a set of representative isolates of *P. haemolytica* and *P. trehalosi* from cattle, sheep and goats (Table 9) were evaluated for their interactions with the different ruminant transferrins. All of these strains were capable of utilizing bovine, caprine or ovine transferrin as a source of iron for growth (data not shown). Immobilized, iron-deficient cells were positive for binding all three of the transferrins in a solid-phase binding assay (Figure 25) and the three transferrins were equally effective at blocking binding to the cells in reciprocal competition binding assays (not shown). In addition, both TbpA and TbpB, molecular weights 100Kda and 60Kda respectively were effectively isolated by affinity resins containing immobilized bovine (Figure 26, Panel A), caprine or ovine transferrin (not shown). These results indicate that the specificity of transferrin-binding within this group of related strains is indistinguishable.

Immunological analysis of transferrin receptor proteins. The observation that bovine, caprine and ovine transferrins compete for the same receptors suggested that there is conservation at least in the binding domain of the receptors. However, the extent of similarity between the individual receptor proteins from different serotypes was not known. To address this question, antibodies were raised against TbpA and TbpB individually and as a complex in rabbits using purified receptor proteins (TbpA and TbpB from a bovine strain h44). These antisera were then tested against receptor proteins from representative strains of different serotypes including isolates obtained from cattle, sheep and goats.

The results in Figure 26, Panel B demonstrate that the antisera raised against purified TbpA and TbpB receptors from *P. haemolytica* serotype A1, strain h44, reacted strongly with similar purified receptors from all of the representative strains. Since there was variation in

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the yield of receptor proteins obtained from the various strains (Figure 26, Panel A), this likely accounts for the slight differences observed in the reactivity of the antisera for a few of the strains (Figures 26, Panel B). Thus, these results suggest that both receptor proteins are conserved amongst the different serotypes of *P. haemolytica* causing pasteurellosis in cattle, sheep and goats.

Although this analysis indicates that there are crossreactive epitopes on the transferrin receptor proteins it does not provide any information as to whether there are crossreactive epitopes at the cell surface where they would be accessible to host immune effector mechanisms. As a first step in attempting to address this question the reactivity of intact iron-deficient cells from the different species to monospecific anti-receptor antisera was tested (Figure 27). These experiments demonstrated that the monospecific antisera prepared against TbpA and TbpB from a type A1 strain of *P. haemolytica* reacted with type A1 strains (h44 and h196, Figure 26, Panel B), several other A serotypes (h98, h103, h105, and h107), and reacted moderately with several *P. trehalosi* strains (h99, h100, h106). The monospecific antisera prepared against TbpA and TbpB from strain h44 reacted strongly with iron-limited whole cells from an extended collection of type A1 strains (data not shown), two of which (h44, h196) are illustrated in Figure 27. There were varying degrees of reactivity with the other serotypes within *P. haemolytica* (h98, h103, h105 and h107) and *P. trehalosi* (h99, h100 and h106). The correlation between the signal obtained with the labelled bTf (Figure 27) and with the anti-TbpA and anti-TbpB antisera (Figure 27) in the different strains suggests that the observed reactivity in intact cells is primarily due to the receptor proteins. The absence of reactivity observed when control antiserum is utilized (data not shown) and the reduced reactivity in iron deficient cells (Figure 26, Panel C) also supports this conclusion.

Genetic analysis of transferrin receptor protein genes. As a complement to the immunological studies the variability of the *tbp* genes from the various strains of *P. haemolytica* and *P. trehalosi* was evaluated. Using the sequence information obtained for the *tbpA* gene from a serotype A1 strain (28), specific primers were prepared for the 5 and 3 ends of the gene (primer #255 and #256, Table 10). These primers were capable of amplifying the intact *tbpA* genes from all the tested strains although only small yields were consistently obtained for strain h100. The intact genes (except from h100) were then subjected to digestion by the *Sau3A* restriction endonuclease and the resulting fragments were analyzed by electrophoresis on polyacrylamide gels. A specific pattern was observed in type A1 strains (h44 and h196, Figure 28, Panel A) and was identical in all the seven type A1 strains that were tested (data not shown). This pattern was also present in most of the other serotypes within *P. haemolytica* (h105, h104, and h98, Figure 28). Subtle changes in the pattern, involving one or more fragments, that is likely attributed to alteration of a single site, was observed for other type A strains (h103 and h107) and within the T strains (h106 and h99).

The restriction digestion analysis was unable to identify any differences between the *tbpA* genes in the A1 isolates. Thus, an alternative approach was adopted for rapidly and simply examining variation amongst the *tbpA* genes. This approach is based on the observation that there are segments of the TbpA (and LbpA) protein, proposed to be surface loops, which show the greatest variation in amino acid sequence among proteins from different species (21 and Legrain et al.1996). In particular, there is one large predicted loop, demonstrated to be at the surface by reactivity with a monoclonal antibody (21), in which the greatest variation in amino acid sequence was observed when aligning TbpAs from two meningococcal strains, one gonococcal strain and four *H.influenzae* strains (1; Loosemore et al.1996;Schryvers and Gonzalez 1996). An oligonucleotide primer was prepared (#450, Table 10) based on the known amino acid sequence (VEDTCPTLD) in this region for *P. haemolytica* typeA1 and used it in colony PCR amplification reactions in combination with a 5 specific primer (#255 , Table 10) with the various strains. As illustrated in Figure 29, Panel A, this oligonucleotide pair readily amplified the anticipated 800 bp PCR product under high stringency conditions from all of the strains except strain h100 where a comigrating band was barely discernable. These results suggest that there is considerable homology amongst the different serotypes of *P. haemolytica* and *P. trehalosi* even in the non-conserved' amino acid regions in TbpA.

In order to evaluate the variation in the *tbpB* genes, a first attempt was made to amplify the intact genes from the various strains using specific primers for the 5 and 3 ends of the gene (primer# 401 and #199, Table 10). These primers readily amplified the intact *tbpB* gene from all the strains tested (Table 11). Restriction enzyme digestion analysis revealed that an identical digestion pattern was observed for all the seven A1 strains tested (see h44 and h196 in Figure 28, Panel B) and some strains from the other groups (h105, h104 and h98). Only subtle differences in the pattern were detected in several of the other strains (h103,h107, h99, h106, Figure 28, Panel B). Thus PCR-based approaches were used with oligonucleotide primers to variable regions to see if the variable domains found in other studies are subject to variation in *P. haemolytica*. A reverse oligonucleotide primer (#397, Table 10) was tested outside the reported conserved regions in *tbpB* (26) in combination with the 5' primer (#401, Table 10). Similarly, a forward primer (#400) from another variable region in combination with the 3 terminal oligonucleotide primer (#199) was used. The anticipated *tbpB* partial products were obtained for all the A and T strains tested except one T strain, h99 (Fig 29B and Table 11) indicating that a considerable homology does occur not only at the 5' and 3' termini of the gene but also in a number of regions known to be variable in other species.

Table 10: Oligonucleotide primers.

#	Description	Direction	Sequence
255	<i>tbpA</i> - 5' end, NdeI site at start codon	forward	CCCTATCATATGATAATGAAATATCATC
256	<i>tbpA</i> - 3' end, HindIII site after stop	reverse	TAGCGCAAGCTTCTAAAACTTCATTTCAAAT

450	<i>tbpA</i> - variable region	reverse	TAATGTTGGGCAAGTATCTTCCAC
401	<i>tbpB</i> - 5' end	forward	TAAATTAAAGGAGACATTATGTTTAAACT
199	<i>tbpB</i> - 3' end, flanking HindIII site	reverse	GCGCAAGCTTTTATTTTCTATTGACG
397	<i>tbpB</i> - variable region, near 3' end	reverse	CTGTTGGCAAATCTGCCAGAG
400	<i>tbpB</i> - variable region, near middle	forward	AGGTAATCGCTTTTCTGGTAAAGC

* Direction relative to orientation of coding strand for the relevant gene

Table 11. PCR-amplification of *tbpA* and *tbpB* gene segments from different serotypes of *Pasteurella haemolytica*.

Strain	Amplification of <i>tbpA</i> gene segments by oligo pair:		Amplification of <i>tbpB</i> gene segments by oligo pair:		
Strain	Primer pair/product amplified		Primer pair/product amplified		
	255/256	255/449	401/399	401/199	400/199
h44	+	+	+	+	+
h93	+	+	+	+	+
h94	+	+	+	+	+
h95	+	+	+	+	+
h96	+	+	+	+	+
h97	+	+	+	+	+
h196	+	+	+	+	+
h99	+	+	-	+	-
h100	+	+/-	+	+	+
h103	+	+	+	+	+
h104	+	+	+	+	+
h105	+	+	+	+	+
h106	+	+	+	+	+
h107	+	+	+	+	+
h174	+	+	+	+	+

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Key:

- + = Product of anticipated size, comparable to the control (h196) obtained
- +/- = Product of anticipated size but much weaker in intensity than the control
- = No product obtained.

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Discussion

A collection of *P. haemolytica* and *P. trehalosi* isolates from other ruminants were found to be capable of acquiring iron from bovine, ovine and caprine transferrins (data not shown) which is presumed to be mediated by surface receptors that specifically bind ruminant transferrins (Al-Sultan and Aitken 1984). The collection of *P. haemolytica* (biotype A) and *P. trehalosi* (biotype T) strains involved in this study includes different serotypes (Table 9), suggesting that the receptor-mediated iron acquisition mechanism is fairly widespread within these species. There was some variability in the expression of the transferrin binding activity amongst the various strains tested under the standard conditions used for the binding assay. Similar variation in the ability to bind the conjugated host transferrin (HRP-hTf) has been observed amongst *H. influenzae* strains (Robki et al.1993). This may be partly attributable to the varying growth characteristics of the different strains. However, the highly sensitive and specific nature of the binding assay enabled definitive identification of the presence of receptor activity in the strains tested.

The competitive binding experiments and the affinity isolation experiments indicate that interaction with all three ruminant transferrins is mediated by the same receptor proteins which show considerable similarity amongst the various strains analyzed. This conclusion is further substantiated by the immunological analysis of receptor proteins prepared from a variety of *P. haemolytica* strains with monospecific sera against TbpA and TbpB (Figure 26, Panel B). Since the immunological analysis included a variety of different serotypes within biotype A and T, it would appear that the transferrin receptor proteins are fairly conserved among *P. haemolytica* disease isolates from ruminants. Although there is considerable immunological cross-reactivity amongst the two receptor proteins in a variety of different *P. haemolytica* strains, this cross-reactivity should include epitopes present at the surface *in vivo*. Preliminary results with the whole cell/antibody analysis (Figure 27) suggest the presence of such surface epitopes. The consistent pattern observed for whole cell/HRP-btf and whole cell/anti-Tbp reactivities for any given strain (Figure 27) coupled with the greatly reduced reactivity observed for both reactions in the iron replete cells suggest that the reactivity observed is against iron regulated proteins.

An important consideration in the development of an effective vaccine against any bacterial species, is the spectrum of the vaccine against the different serotypes/biotypes of the infecting bacterial species. In *N. meningitidis* while TbpA proteins are relatively homogenous, the identification of two families in the species based upon the differences in the

molecular masses and antigenic properties of their TbpB proteins (18) dictates the formulation of a heterogenous Tbp vaccine representative of the two families.

The 100Kda transferrin receptor protein (TbpA), from bovine strain of *P. haemolytica*, serotype A1 (26) was identified by the affinity purification procedure of Schryver and Morris (32). Modification of that procedure, as described in the method section, has enabled us to identify the 60Kda as the second transferrin binding protein in *P. haemolytica* analogous to TbpB in the other bacterial species (32 and Robki et al.1993). The affinity purification procedure isolated TbpA and TbpB of similar molecular weights from all of the strains of *P. haemolytica* and *P. trehalosi* examined (Figure 26, Panel A). Our results in *P. haemolytica* and *P. trehalosi* showed that antisera raised against these two purified receptor proteins from a serotype A1, strain h44, specifically recognised the receptor proteins from other A1 strains (h196, Figure 26, Panel B) and other A serotypes. These included an A11 serotype (h107) known to differ from the other A types in its reactivity with convalescent sera against the 35Kda and 70Kda iron regulated outer membrane proteins. The amounts of purified receptors from the T strains, h99 and h100 (Figure 26, Panel A) and their with the antisera (Figure 26, Panel B) appeared to be reduced compared with most of the A strains. This reduction in the antibody reactivity with the T strains was also observed in whole cell assay (Figure 27). The reduction in the whole cell-antibody reactivity in these strains, however, was consistent with their reduced reactivity with HRP-bTf (Figure 27). This suggests that the observed lower reactivity with the anti-TbpA and anti-TbpB antisera is likely due to differences in the expression of receptor proteins under the standard growth conditions used in these experiments.

The genes encoding TbpA and TbpB proteins, *tbpA* and *tbpB* are fairly homogeneous within serotype A1 (causing pneumonic pasteurellosis in cattle) and to a large extent, amongst the A types (causing pneumonia in sheep and goats) in general (Figure 28). By extending the restriction enzyme digestion analysis to the *P. trehalosi* strains, we found only marginal differences (Figure 28). The results of PCR- amplification experiments with specific 5' and 3' oligonucleotide primers and primers derived from the hypervariable regions (Figure 29, Table 11) further supports the conclusion that the *tbp* genes within *P. haemolytica* species and *P. trehalosi* are relatively homogenous. These results contrast with the differences which have been observed for *H. influenzae* and *N. meningitidis* (1 and Loosemore et al.) but will need to be confirmed by sequence analysis of genes isolated from representative strains from a number of serotypes.

The lack of evident genetic heterogeneity in the *tbp* genes and the apparent immunological crossreactivity of the Tbp proteins from a variety of *P. haemolytica* and *P. trehalosi* strains underscore their potential as broad-spectrum vaccine antigens for prevention of infection in ruminants.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not

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- 5 All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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DETAILED FIGURE LEGENDS

Figure 1. Result of PCR analysis

- a) Schematic diagram of the PCR procedure - Each circle represents a recombinant pBR322 plasmid and a possible PCR reaction. The Tbp1 primer, primer left and primer right are represented by the letters t, l and r respectively. The *EcoRI* site on the pBR322 plasmid is denoted by the letter, E. In the upper plasmid, the Tbp1 primer and primer left would amplify a PCR product corresponding to the heavy line. Similarly in the lower plasmid, the PCR product amplified by Tbp1 primer and primer right is represented by the heavy line.

(b) The 0.8 kb PCR product amplified by Tbp1 primer and primer left.

- 10 **Figure 2.** Restriction endonuclease map of *tbp* plasmids 9, 10 and 482. Open box - pBR322 presented linearly. Crosshatched box - PCRII presented linearly. The positions and the orientations of *tbpA*, *tbpB* are as shown by the dark arrows.

Figure 3. Preliminary nucleotide sequence of *P. haemolytica* *tbpA* and *tbpB*. Putative signal sequence cleavage sites are indicated by an arrow. The start codon (ATG) is underlined.

- 15 **Figure 4.** The promoter region of *P. haemolytica* *tbpB* (PHTBPB). The putative Fur consensus sequence is indicated by asterisks. The Fur consensus sequences of *N. gonorrhoeae* *tbpB* (NGTBPB) and *N. meningitidis* *tbpB* (NMTBPB) are also indicated.

- Figure 5.** Southern hybridization of *P. haemolytica* genomic DNA digested with *ClaI* and probed with the *tbpA* gene. Lanes 1-16 represent *P. haemolytica* serotypes 1 to 16. Lane M represents lambda DNA digested with *Hind III* and hybridized with lambda DNA radiolabelled separately as size markers.

Figure 6. Southern hybridization of *P. haemolytica* genomic DNA digested with *HindIII* and *BamHI* and probed with the *tbpA* gene. Lanes 1-16 represent *P. haemolytica* serotypes 1-16. The molecular sizes are as indicated on the left.

- 25 **Figure 7.** Southern hybridization of *A. suis* 3714, *A. pleuropneumoniae* CM5 and Shope 4074 genomic DNA digested with various restriction endonucleases and probed with *P. haemolytica* *tbpA*. Lane M represents lambda DNA digested with *Hind III* and hybridized with lambda DNA radiolabelled separately as size markers.

- Figure 8.** Restriction maps of the *tbpA*, *tbpB* regions in *P. haemolytica* A1, *A. pleuropneumoniae* CM5, Shope 4074 and *A. suis* 3714. Line 1 represents the *tbpA* probe used in Figure 15. Line 2 represents the *tbpA* probe used in Figures 16 and 17.

- Figure 9.** Alignment of the amino acid of Tbp1 of *P. haemolytica* A1 (PHTBP) and the Tbp1 of *N. gonorrhoeae* (NGTBP1) and *N. meningitidis* (NM1). The numbers to the right indicate amino acid positions. Asterisks indicate positions of complete identity in alignment, dots indicate similar amino acid residues. Gaps were introduced to maximize sequence alignment and are indicated by dashes (-).

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Figure 10. Alignment between *P. haemolytica* A1 Tbp1 (PHTBPI) and the *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7). Asterisks indicate positions of complete identity in alignment, dots indicate similar amino acid residues. Gaps were introduced to maximize sequence alignment and are indicated by dashes (-).

- 5 **Figure 11.** Dendrogram illustrating the genetic relatedness among *P. haemolytica* Tbp1 (PHTBP), *N. gonorrhoeae* Tbp1 (NGTBP1), *N. meningitidis* Tbp1 (NM1) and the TfbA proteins from *A. pleuropneumoniae* serotype 1 and 7 (APL1, APL7).

- Figure 12.** Peptide alignment between *P. haemolytica* A1 Tbp1 and TonB-dependent outer membrane receptors of *E. coli*. Asterisks show amino acids with complete identity in alignment, dots indicate similar amino acid residues. Gaps were introduced to maximize sequence alignment and are indicated by dashes (-).
- 10

Figure 13. T7 analysis of the *P. haemolytica* Tbp1 protein. The molecular weight marks in kDa are as indicated on the left. Lane 1 - positive control recombinant plasmid. Lane 2 - the recombinant plasmid containing *tbpA*. Lane 3 - the vector plasmid pBluescript (SK)

- 15 **Figure 14.** Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E. coli* HB101. The first antibody was a rabbit antiserum raised to the soluble antigens of *P. haemolytica* A1 and the second antibody was goat anti-rabbit alkaline phosphatase conjugate. Lane M represents the molecular weight markers in kDa. Lanes 1-4 represent outer membrane fractions and lanes 5-8 are inner membrane fractions. 6 µg of protein was added to each lane.
- 20 Lanes 1 and 5 - *E. coli* proteins from cells grown in LT. Lanes 2 and 6 - proteins from cells grown in BHIB. Lanes 3 and 7 - proteins from cells grown in BHIB plus 100 µM EDDA. Lanes 4 and 8 - proteins from cells grown in BHIB plus 100 µM EDDA with 1 mM FeSO₄ added.

- Figure 15.** Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E. coli* HB101 using sera raised in calves to soluble antigens by vaccination with Presponse®. The second antibody was goat anti-bovine alkaline phosphatase conjugate. Lane M represents the molecular weight markers in kDa. Lanes 1-4 are outer membrane fractions and lanes 5-8 are inner membrane fractions. 6 µg of protein was added to each lane. Lanes 1 and 5 - *E. coli* proteins from cells grown in LT. Lanes 2 and 6 - proteins from cells grown in BHIB. Lanes 3 and 7 - proteins from cells grown in BHIB plus 100 µM EDDA. Lanes 4 and 8 - proteins from cells grown in BHIB plus 100 µM EDDA with 1 mM FeSO₄ added.
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- Figure 16.** Binding of labelled transferrins by iron-deficient bacterial membranes. Aliquots of total membranes (4 µg protein) prepared from iron-deficient cells from the indicated bacterial strains were spotted onto strips of nitrocellulose/cellulose acetate paper and, after blocking, the papers were exposed to mixtures containing 450 ng/ml of the indicated HRP-conjugated transferrin. The filters were subsequently washed and developed with HRP substrate mixture as described in the Methods section. h173, h174, h175, h176 and h44 are representative strains of *P. haemolytica* whose serotype and source are listed in Table 1. h50 - *A. equuli*. HRP-bTf, -oTf, -cTf and -eTf - HRP conjugates of bovine, ovine, caprine and equine transferrins.
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Figure 17. Isolation of receptor proteins with transferrin affinity columns. Affinity isolation experiments were performed with iron-deficient total membranes prepared from *P. haemolytica* strain h44 (top panel), h173 (middle panel) and h175 (bottom panel). Experiments were performed with bovine transferrin-Sepharose (lanes A and B), ovine transferrin-Sepharose (lane C), caprine transferrin-Sepharose (lane D) or equine transferrin-Sepharose (lane E) using standard washing conditions (lanes B-E) or low salt washing conditions (lane A) as outlined in the methods section. The samples eluted with buffer containing 2M guanidine HCl were dialyzed, concentrated and aliquots analyzed by SDS-PAGE and silver staining as described in the methods section.

Figure 18. Immunological analysis of receptor proteins from different serotypes of *P. haemolytica* from bovine, sheep and goats. Aliquots of purified receptor proteins from representative serotypes of *P. haemolytica* were subjected to SDS-PAGE, electroblotted and then probed with specific anti-TbpB serum (Panel A) or with anti-TbpA serum (Panel B) as described in the methods section. The following *P. haemolytica* strains of the indicated serotype were included in the analysis; Lane 1 - strain h44 (A1), Lane 2 - h173 (untypable), Lane 3 - h175 (A7), Lane 4 - h176 (A9), Lane 5 - h100 (T4), Lane 6 - h106 (T10), and Lane 7 - h107 (A11). The numbers on the left represent the molecular weights (X 1000) of standard proteins.

Figure 19. Binding of labelled transferrin and anti-receptor antibody by intact cells. The indicated bacterial strains were grown under iron-limiting conditions, harvested by centrifugation and resuspended to a A_{600} of 1-2 in 50 mM TrisHCl, 150 mM NaCl, pH 7.5 buffer. A 5 μ l aliquot of the suspensions were applied to HA membrane, the membrane was dried, blocked and then exposed to blocking solution containing labelled transferrin (HRP-bTf) or anti-receptor antibody (anti-TbpA, anti-TbpB). The latter membranes were washed and subsequently exposed to labelled second antibody prior to development with substrate.

Figure 20. Map of the *P. haemolytica* *tbp* operon (Top) and regulatory sequences (Bottom). *tbpA* and *tbpB* are the genes encoding for TbpA and TbpB, respectively; *p*, is the putative promoter region preceding *tbpB* and denoted as -35 and -10 sites at the bottom. A putative Fur box is represented as two arrows in opposite directions in the sequence at the bottom; *rnaseT* and *fis* are two ORFs flanking the *P. haemolytica* *tbp* operon encoding for proteins highly homologous to *E. coli* and *H. influenzae* RNase transferase and factor-for-inversion stimulation proteins, respectively. Additionally, putative ribosomal binding site or Shine-Dalgarno (SD) consensus sequence, transcriptional start (Met), and stop codons (SC) are also bolded.

Figure 21 . The DNA sequence of the *tbpA* gene from *P. haemolytica* strain H196.

Figure 22. Predicted amino acid sequence of the TbpA protein from *Pasteurella haemolytica* strain H196. Italicized amino acids correspond to the experimentally determined N-terminal amino acids of the mature protein. Residues indicated by strikethrough constitute the leader peptide region. Residues that are identical in TbpAs from *Neisseria meningitidis*, *N. gonorrhoeae*, *H. influenzae* and *Actinobacillus pleuropneumoniae* are bold and underlined.

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Regions proposed as internal segments (dark shading), intermembrane b-strands (light shading) or external segments (no shading) based on the proposed topology model by Tommassen (28) are indicated.

Figure 23. The DNA sequence of the *tbpB* gene from *P. haemolytica* strain H196.

- 5 **Figure 24.** Predicted amino acid sequence of the TbpB protein from *P. haemolytica* strain H196. Italicized amino acids correspond to the predicted N-terminal amino acids of the mature protein. Residues indicated by strikethrough constitute the leader peptide region. Regions of homology are identified by shading and residues that are identical in TbpBs from *Neisseria meningitidis*, *N. gonorrhoeae*, *H. influenzae* and *Actinobacillus pleuropneumoniae* are bold and underlined.

- 10 **Figure 25.** Solid-phase HRP-Tf binding assay. Total membrane preparations from the indicated strains of *P. haemolytica* and *P. trehalosi* were spotted onto nitrocellulose-cellulose acetate paper and blocked with skim milk prior to incubation with HRP-bTf, -oTf or -gTf. Binding was detected with chloronaphthol reagent as in the methods. The letters on the left indicate the strains while the letters on the top indicate the different HRP-labelled ruminant transferrins.

- 15 **Figure 26.** Western blot cross-reactivity studies with anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1. Aliquots of affinity purified receptor proteins from the indicated strains of *P. haemolytica* and *P. trehalosi* were separated by SDS-PAGE and Silver stain (panel A) or subjected to Western blotting (panel B) as in Methods. Tbp proteins were identified by incubation with a mixture of anti-TbpB(1/1000) and anti-TbpA(1/1000) rabbit antisera as described in the methods section. The numbers on the left represent the molecular weights (X 1000) of standard proteins in kilodaltons.

- 20 **Figure 27.** Cross-reactivity studies with monospecific anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1 against intact cells. Aliquots of iron-limited intact cells from the indicated strains of *P. haemolytica* and *P. trehalosi* were spotted onto HA nitrocellulose paper and, after blocking, the membrane was either exposed to HRP - labelled bTf, anti-TbpA antiserum or anti-TbpB antiserum. Bound antibodies were subsequently detected by labelled goat anti-rabbit antibody as described in the methods. A preparation of intact *P. haemolytica* strain h44 (type A1) cells grown under iron-sufficient cells (indicated by h44 - Fe⁺) was spotted
- 25 onto the membranes and used as a control.

- 30 **Figure 28.** Restriction endonuclease digestion pattern of PCR-amplified *tbpA* (Panel A) and TbpB (Panel B) genes from *P. haemolytica* and *P. trehalosi* strains. The *tbp* genes amplified by colony PCR from the indicated strains were digested with Sau3A1 restriction endonuclease. The resulting digests were electrophoresed on a 7.5% polyacrylamide gel as described in Methods.
- 35 The letters above the lanes indicate the source strain template DNA used in PCR while the letters on the left indicate the molecular weight standard in kilobases. Imaging was done with a Hewlett-Packard ScanJet IIP. In fig 29B, primer #s 397 and 400 from non conserved regions of

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P.hamolytica *tbpB* gene, were used in combination with opposing primers (#s 401(5') and 199(3')) respectively.

Figure 29. PCR amplification of variable segments of the *tbpA* and *tbpB* genes. For *tbpA* gene, oligonucleotide primer # 450 made from the deduced amino acid sequence from a hypervariable region of *tbpA* was used in combination with the 5' specific primer (#255) to amplify the gene segment from the various *P.haemolytica* strains . The products were then analysed on 1% agarose gel followed by staining with ethidium bromide.

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WE CLAIM:

1. A purified and isolated nucleic acid molecule comprising a sequence encoding a transferrin binding protein of *P. haemolytica*.
2. A purified and isolated nucleic acid molecule according to claim 1 comprising a sequence
5 encoding TbpA of *P. haemolytica*.
3. A purified and isolated nucleic acid molecule as claimed in claim 2, which encodes a protein having the amino acid sequence as shown in Figure 22 and SEQ ID NO:2.
4. A purified and isolated nucleic acid molecule as claimed in claim 2, having a sequence which comprises (a) a nucleic acid sequence as shown in Figure 21 and SEQ ID NO:1 (b) nucleic
10 acid sequences complementary to (a); (c) nucleic acid sequences which are at least 80% homologous to (a); or (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.
5. A purified and isolated nucleic acid molecule according to claim 1 comprising a sequence encoding TbpB of *P. haemolytica*.
- 15 6. A purified and isolated nucleic acid molecule as claimed in claim 5, which encodes a protein having the amino acid sequence as shown in Figure 24 and SEQ ID NO:4.
7. A purified and isolated nucleic acid molecule as claimed in claim 5, having a sequence which comprises (a) a nucleic acid sequence as shown in Figure 23 and SEQ ID NO:3; (b) nucleic acid sequences complementary to (a); (c) nucleic acid sequences which are at least 80%
20 homologous to (a); or (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.
8. A naturally occurring nucleic acid molecule which is characterized by the ability to hybridize to the purified and isolated nucleic acid molecule as claimed in claim 2 under stringent hybridization conditions.
- 25 9. A naturally occurring nucleic acid molecule which is characterized by the ability to hybridize to the purified and isolated nucleic acid molecule as claimed in claim 5 under stringent hybridization conditions.

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10. An oligonucleotide comprising at least 15 contiguous bases of a nucleic acid molecule as claimed in claim 2 which is characterized by the ability to hybridize to the nucleic acid molecule under stringent hybridization conditions.
11. An oligonucleotide comprising at least 15 contiguous bases of a nucleic acid molecule as
5 claimed in claim 5 which is characterized by the ability to hybridize to the nucleic acid molecule under stringent hybridization conditions.
12. A recombinant expression vector adapted for transformation of a host cell comprising a nucleic acid molecule as claimed in claim 2 and one or more transcription and translation elements operatively linked to the nucleic acid molecule.
- 10 13. A recombinant expression vector adapted for transformation of a host cell comprising a nucleic acid molecule as claimed in claim 5 and one or more transcription and translation elements operatively linked to the nucleic acid molecule.
14. A host cell containing a recombinant expression vector as claimed in claim 12.
15. A host cell containing a recombinant expression vector as claimed in claim 13.
- 15 16. A purified and isolated TbpA protein.
17. A purified and isolated TbpA protein as claimed in claim 16, which has the amino acid sequence as shown Figure 22 and SEQ ID NO:2, or fragments thereof capable of binding transferrin.
18. A purified and isolated TbpB protein.
- 20 19. A purified and isolated TbpB protein as claimed in claim 18, which has the amino acid sequence as shown in Figure 24 and SEQ ID NO:4, or fragments thereof capable of binding transferrin.
20. A method for preparing a TbpA protein comprising (a)transferring the recombinant expression vector according to claim 12 into a host cell; (b) selecting transformed host cells from
25 untransformed host cells; (c) culturing a transformed host cell under conditions which allow expression of TbpA ; and (d)isolating the recombinant TbpA.

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21. A method for preparing a TbpB protein comprising (a)transferring the recombinant expression vector according to claim 13 into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a transformed host cell under conditions which allow expression of TbpB ; and (d)isolating the recombinant TbpB.
- 5 22. A polyclonal or monoclonal antibody having specificity against an epitope of TbpA or TbpB.
23. A vaccine for the prophylaxis and treatment of an infection caused by a *Pasteurella* spp., wherein said vaccine comprises an immunologically effective amount of at least one of TbpA and TbpB of *P. haemolytica* and a pharmaceutically acceptable carrier.
- 10 24. The vaccine according to claim 23 wherein said infection is caused by *Pasteurella haemolytica*.
25. A vaccine for the prophylaxis and treatment of an infection caused by a *Pasteurella* spp., wherein said vaccine comprises an immunologically effective amount of TbpA and TbpB and a pharmaceutically acceptable carrier.
- 15 26. A vaccine for the prophylaxis and treatment of an infection caused by a *Pasteurella* spp., wherein said vaccine comprises an immunologically effective amount of TbpB and a pharmaceutically acceptable carrier.
27. The vaccine according to claim 26 wherein said TbpB has the amino acid sequence as shown in Figure 24 and SEQ ID NO:4.
- 20 28. A vaccine for the prophylaxis and treatment of an infection caused by a *Pasteurella* spp., wherein said vaccine comprises an immunologically effective amount of a recombinant expression vector according to claim 12 and a pharmaceutically acceptable carrier.
29. A vaccine for the prophylaxis and treatment of an infection caused by a *Pasteurella* spp., wherein said vaccine comprises an immunologically effective amount of a recombinant
25 expression vector according to claim 13 and a pharmaceutically acceptable carrier.

BP File No. 6580-057

ABSTRACT OF THE DISCLOSURE

Novel transferrin binding proteins from *Pasteurella haemolytica*, and nucleic acid molecules encoding the novel proteins are disclosed. Antibodies against the novel proteins are disclosed. The invention also relates to vaccines containing the novel proteins of the invention. 5 The invention also provides methods for identifying substances which affect the binding of transferrin to the proteins and methods for screening for agonists or antagonists of the binding of the proteins and transferrin.

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JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below next to our names;

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled **TRANSFERRIN BINDING PROTEINS OF PASTEURELLA HAEMOLYTICA AND VACCINES CONTAINING SAME** the specification of which:

☒ [X] is attached hereto.

☐ [] was filed on _____ as application serial number _____ and, if applicable, was amended on _____.

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application Number	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed Under 35 U.S.C. 119
				Yes <input type="checkbox"/> No <input type="checkbox"/>
				Yes <input type="checkbox"/> No <input type="checkbox"/>
				Yes <input type="checkbox"/> No <input type="checkbox"/>

Prior United States Application(s)

We hereby claim the benefit under Title 35, United States Code, § 119 or §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial Number	Date of Filing (day, month, year)	Status - Patented, Pending, Abandoned
60/008,569	December 1, 1995	Pending Provisional

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

Jerry W. Mills, Registration No. 23,005; Scott F. Partridge, Registration No. 28,142; Rodger L. Tate, Registration No. 27,399; James G. Gatto, Registration No. 32,694; James Remenick, Registration No. 36,902; C. Patrick Turley, Registration No. 35,723; Laurence H. Posorske, Registration No. 34,698; Christopher C. Campbell, Registration No. 37,291; James B. Arpin, Registration No. 33,470; Steven P. Klocinski, Registration No. 39,521; Eric Sinn, Registration No. 40,177; and Stacy B. Margolies, Registration No. 39,760.

All correspondence and telephone communications should be addressed to: **James Remenick, Baker & Botts, L.L.P.; The Warner, Suite 1300; 1299 Pennsylvania Avenue, N.W.; Washington, D.C. 20004-2400; (202) 639-7700**, which is also the address and telephone number of each of the above listed attorneys.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature: _____ Date: _____

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Signature: _____ Date: _____

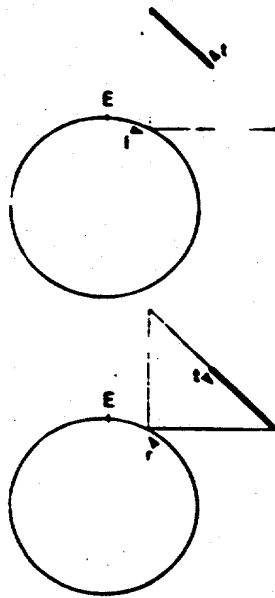
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FIGURE 1



a



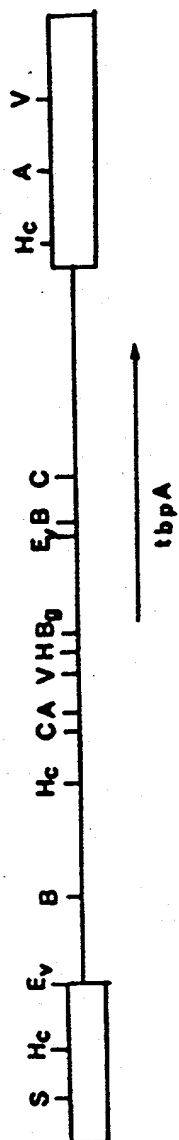
b

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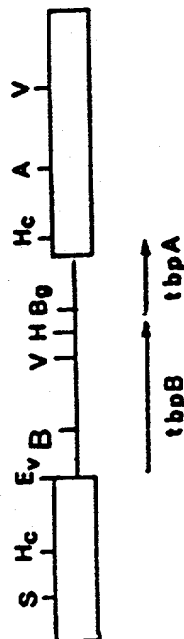
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FIGURE 2

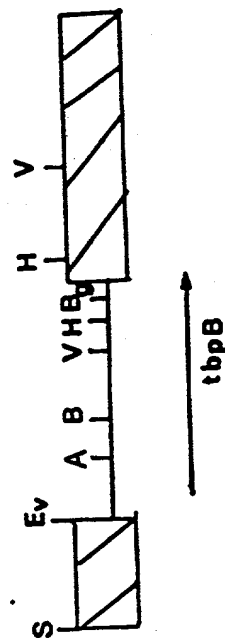
Plasmid 9



Plasmid 10



Plasmid 482



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FIGURE 3

tbpB

	10	20	30	40	50
1	CGCTTGCAGA	TTTGTAAAAA	ATTTAGCTAA	AATCAGACCT	GGCTTGTATT
51	TTAGGGTTAT	<u>TAT</u> GGAACAG	ACAACGGAAC	AAATAGATTA	TAAATTATTA
101	AAGCATCGTT	TTCGTGGCTA	TTTACCGCTT	GTGATTGATG	TGGAACAGAG
151	GCTTAAATGC	CCAAACTGTG	CCTTATTGGA	ATTGGCCGGA	ATTACTTTAA
201	AATTGGACGA	GCCAAGGTTA	TCTTATTGCC	GGATTCAACA	ATGCCAATTT
251	CCAATTTGCG	GCCATTGGAG	GGGGCGATTT	TCAAGGGCCG	AATATTTACC
301	CTCAGTCATT	AAAATTCAAC	GGCATTCTAA	TTCAACAACC	TCTAGAGGAA
351	GCGGTTTAGA	GAATATTGCC	CAATTCCCGG	AAATGTTCAA	AATGGTTCGC
401	AAAGCAATGA	AGGAAGCAAG	GCTGCCAACG	GGCGGTGATT	GTGCCCCAC
451	AATGCCAGCG	TTCGATCAGG	GCTTTTTTTAC	AGGGCTGCGA	TTAAACGGAA
501	TGCCAAGCGA	GAGATCCCTT	TCACCCTTTT	GGCGATGTTT	GACTACCGCC
551	GCCACCCTTT	GCAGGTTTTA	TGTTATGGGC	AAACCGTGTT	CGTTAAAAGC
601	CTGCCAGTGC	AAAATCCGTT	CACGTAGCAG	CACTATAGGG	CGAATTGGGT
651	ACCGGGCCCC	CCTCGAGGTC	GACGGTATCG	ATAAAGCTTC	ATATCGAATT
701	CCTGCAGCCC	GGGGGATCCG	ATGCGCCTTG	CGGCTCAAGT	TATTAGTGGC
751	ATCGGTTTTT	TGGGTGATGG	TGTCATTTTG	CATAAGAAAA	ATGATGCGAT
801	TTCAGGTTTA	CCCACTGCGG	CGATTATTTG	GGCTTCTGCG	GGGATCGGTA
851	TTGCTGCAGG	GGAGGGTTTC	GTGTTTCATG	CGGTCATCGC	CACTGTCATT
901	ATTTTGGTGT	CTATTCGATT	ATGTCCGTTG	GTTCAACGTT	GGGTTCATCG
951	TAAATCACAA	CGTCGTCGAC	GAAATATTCT	TGTCAATGAT	GCGGAAAGCA
1001	TACGGAAAGT	TACCCAATTG	TTATTCAATA	ATCAGTATCG	TATTGAACAT
1051	ATACAAGTCA	AAGATCAAAG	TAGTGGAGAA	GTTGCCGGTT	ACAAATTCGT

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FIGURE 3 CONT'D

1101 ATTGATTCCA CAATGTTCAA AGATGCGTAT GCTTTACTTA AAGCAGAAGA
1151 TGGCGAATAC TGTTCAGTA GATATCATGA AAAAGAGTGC TTATATTCAA
1201 TTTTTTATTT TTTATTTAAT TTCTTTCCAC AAAAGATCAT TTTCAATTAT
1251 ATATACTGGA ATTTTGCCTT ACGCTATCTG TCATTTATTA TGCTATTCAA
1301 GCACAACAAA CTATGGAACA ACAATCAAAA TGTACGCTTA TCGGCTGCCG
1351 ATGATTTCGA TAATGATCGA TGTGCAGAAA TATTTGAAC TACGATTTTC
1401 ACTGAGCAGT CAAAGCACGT TCGCGAGTAT CGACTCTACT TTATTTATCG
1451 TTTGTGCACT ATGTATAATC CACCTAATTC CGTGCCTTGG CCATAAAAGC
1501 CCCCTTCAAA TTGTATTTAT ATCAGCTACC GTGCCACCAT TCGTACTTTT
1551 CGGATCAAGA TTAAAACAGA ATCCCTGCAT GCACAGCGAA ATCAGCTGCG
1601 GTATGCGAAA CGCCGCAGGA GCGCAGTACG CGAAGTGTAC CGTCACTATC
1651 AGTGCTAGAT TTGTCAATAA AAAATTAGTG ACCAAGCTTG GGTGCATAAT
1701 GATGGTGATG AAAGAACGCT CAATGCTTGA CACGTTGCAG GCTATCTGTA
1751 AGGGTATGGT AGTTACAGGC ACAGCCCAA CGGCCAATTG CTGGTTTTTT
1801 ATCCTTGATC CGAACAGTAC GAATGGTGGT CACGGTAGTG CATATACATA
1851 CTCAATTGAA GGGGGCTTTT ATGGCCCTAA GGCGACGGAA TTAGGTGGTA
1901 TTGTACATAG TGCAGAAACG GATAAAGATA GAGTCAGTAT TACATTCGGC
1951 GGAAAACGTC AAATAGAAAA ATAATCATAA TTCCCCTTTG CTGGTTGTAG
2001 ATAGCAGCGG GCAATTTTTT ATAAAAATTT GCAAAATTTA AATAA

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FIGURE 3 CONT'D

tbpa

	10	20	30	40	50
1	AGACCCTATC	TAATGATAAT	GAAATATCAT	CATTTTCGCT	ATTCACCTGT
51	TGCCTTAACA	GTGTTATTTG	CTCTTTCTCA	TTCATACGGT	GCTGCGACTG
101	AAAATAAAAA	AATCGAAGAA	AATAACGATC	TAGCTGTTCT	GGATGAAGTT
151	ATTGTGACAG	AGAGCCATTA	TGCTCACGAA	CGTCAAAACG	AAGTAACTGG
201	CTTGGGGAAA	GTAGTGAAAA	ATTATCACGA	AATGAGTAAA	AATCAAATTC
251	TTGGTATTCG	TGATTTAACT	CGCTATGACC	CTGGTATTTT	GGTGGTGGA
301	CAAGGTCGCG	GTGCAAGTAG	TGGCTATGCC	ATTCGAGGTG	TAGATAAAAA
351	CCGTGTCAGC	TTACTTGTTG	ATGGGCTACC	ACCAGCGCAC	AGTTATCATA
401	CGCTGGTTCA	GATGCTAATG	GTGGTGCAAT	TAATGAGATT	GAGTATGAAA
451	ACATTCGTTT	AATTGAGTTA	AGCAAAGGAG	CAAGTTCTGC	GGAATATGGC
501	TCTGGTGCGC	ATGGTGGTGC	TATTGGTTTT	CGTACTAAAG	ATCGCCAGGA
551	TATTATTAAA	GAGGGGCAGC	ATTGGGGCTT	ACATAGTAAG	ACCTCTTATG
601	CCAGCAAAAA	TAGCCATTTT	TACAGTCTAT	CGCAGCGGCT	GGTCAGGCGG
651	GTGGTTTTCA	AGCACTTGTT	ATTGCAACTC	ACCGACACGG	TAAAGAGACC
701	AAAATTCATT	CCGAGGCAAA	TCAATTACAT	ATTATTCGGC	GTATAACCGG
751	CTTTCAAAAT	CGCTACGACT	TTACCCAATT	CCGCACAGAA	TGCCTCCTGG
801	AGGATCTTTT	TTTATTGTGG	AAGATACTTG	CCCAACATTA	GATTGTACTC
851	CTCGTGCAAG	GGTTAAGTTG	AACGCGATAA	TTTCCCAGTC	AGAACATTTT
901	CGAATATACG	CCTGGAAGAG	GCGAAACAGC	TTGAGATTCC	TTATCGCACT
951	GAGCTCTCAG	CCCAAAGAAT	ARACCGGTAA	AGATCGCATT	GCACCAAACC
1001	CTTTAGATTA	CAAGAGTAAT	TCTGTTTTTA	TGAAGTTTGG	CTATCACTTT
1051	ACCTCGTCTC	ATTATCTTGG	CGCATCTCAC	AAGATGATAC	AAAACAACGC
1101	ACGATATCCG	TCATSTGCAA	ACGCCAGCTT	ACTATACAAA	AGACGATATT
1151	TACTTATCAC	TTTGGAAC TA	TGTTTATCAA	GGGGATATTA	TTTAGATGGC
1201	TTAGTGTTCA	AGCCAAGGAT	CCCTTATGGG	TTGCGCATAT	GCCATGTGAA

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FIGURE 3 CONT'D

1251 CGTCACCACA AACGTCGTTT AGGATTCACC TATAAATATA AACCAGAGAA
1301 TAATCGCTGG TTGGATAGCA TTAACCTCGT CGTACGTGCT TTGCGCTCTC
1351 GCTGCTGTGC TCTGAGTAAA CAAGATATTG AACTATATAG CCGGCTACAT
1401 CGCTTGCAAT GTAGCGATTA TCCTGTGGTA GATAAAAATT GCGGCCCGAC
1451 TTTGGATAAA TCTGGTCTAT GTATCGAACT GAGCGTAATA ATTACCAAGA
1501 AAAGCATCGT GTCATTCATT TAGAATTTGA TAAAGCGCTA AATGCTGGTC
1551 AAGGCGTATT TAAGCAAACC CACAACTGA ATTTAGGCTT GGGCTTTGAA
1601 TCGATTAATC GCTTATGATC ATGGGGATAT GACTGCCCAA TATACCAAAG
1651 GCCGGTTATA CCAGCTACCG CGGAGAGGGG CTTTAGATAA TCCATATATT
1701 TATCGCCGCG ATCCACGCAG TATTGAAACG GTATCTTTGT GTAATAATAC
1751 AGCGGCGACA CTTAACTGTG ACGCGTTAAA TAAAGGCATA CGTTTGTACC
1801 TCCGCTGCAC TTAGGAACTA TAGTTTATGA AGGGGATAAT ATTTAGATGG
1851 CTTAGTGTTT AAGCAAGCAA GGATCCCTTA TGGGTTGCGC GATATGCCAT
1901 GTGAAGTTTT TGATGAACGT CACCACAAAC GTCGTTTAGG ATTCACACCT
1951 ATAAATATAT AAACCAGAGA ATAATCGCTG GTTGGATAGC ATTAACCTCGT
2001 GCGTACGTGC TTTGCGCTCT CGCTGCTGTG CTGTGAGTTA AACAAGATAT
2051 TGAAACTTAT AGCCGGCTAC ATCGCTTGCA TTGTGAGCGA TTATCCTGTG
2101 GTAGTAGTAA AAATTGCGGC CCGACTTTGG ATAAATCTTG GTCTATGTAT
2151 CGAACTGAGC GTAATAATTA CCAAGAAAAG CATCGTGTCA TTCATTTAGA
2201 ATTTGATAAA GCGCTAAATG CTGGTCAAGG CGTATTTAAG CAAACCCACA
2251 AACTGAATTT AGGATTGGGC TTTGAATCGA TTAATTCGCT TATGGATCAT
2301 GGGGATATGA CTGCCCAATA TACCAAAGGC CGGTTATACC AGCTAACCGC
2351 GAGAGGGCGT TTAGATAATC CATATTTATC GCCGCGATCC ACGCAGTATT
2401 GAAACGGTAT CTTTGTGTAA TAATACACGC GCGACACTAA CTGTGACGCG
2451 TTAAATAAAG GCATACGTTT GTACCTCCGC TGCTGCCTAA TAAATCAAAA
2501 GAATAACCGA GATACGGTTC AGTGTTGTTC CAACCAGTTG CGATGGCCCA

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FIGURE 3 CONT'D

2551 CTACGTGAAC CATCACCCTA ATCAAGTTTT TTGGGGTCGA GGTGCCGTAA
2601 AGCACTTAAC CCTTCTGTCG TCTCCCGTGG ATGCTTAAAT TCGCAGCTAG

2651 TGGCAGGCAG GCACGTCACT CCTCTCGGTG ATTTCAGGTG CAACTGACCG
2701 GTTCTTGGA CCACCCCTGA TATTAACCGG AGTCAATTAT AAAAACGAGT
2751 TACGTGGAGC GCAATTTATA ATGTCGATGT CAGATACTGT AAAACTCTAT
2801 ATTACCGTGG GCAGCAATTA GGTGACAGGG CCACGGGGCA AGCGAAACCA
2851 GACGGGTACC AATTACACCG ATTTGCCGCC CCCGGGAGAG AAATTTTCAGT
2901 TACCATTCAA AGAAGTTTAG AGCCGGCCAA AAGAAAATAC AAAAAACGCT
2951 GAAAGTATAT TCAGCGCGTT TTTGTTGCTC TAACGGATTA CATACGAATT
3001 CAAAATGTTT TAACGGTCGG TTA

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FIGURE 4

CGCTTGCAGATTGTGTAATAAATTAGCTAAATCAGACCTGGCTTGATTTTACGGTTATTATG PHTBPB

-35

-10
Met

TTTAAATAATAATAATAATAATCCTTATCATTCCTTAAATGATCGGGTTGTATG NGTBPB

-35

-10
Met

GTATTGCAATGTGTAATAATAATAATAATCCTTATCATTCCTTAAAT TGAATTGGGTTTATG NMTBPB

-35

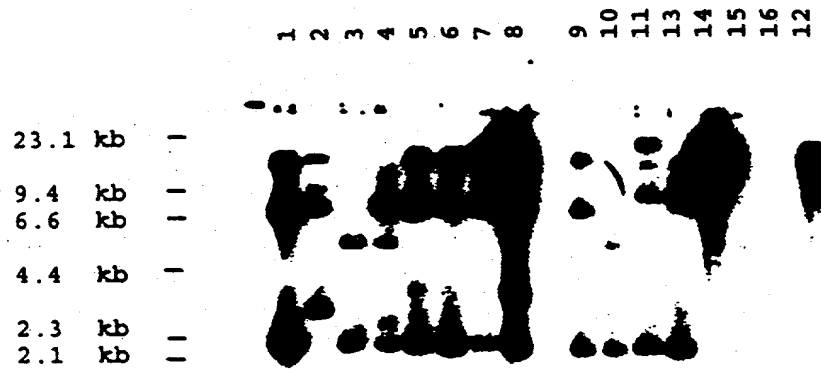
-10
Met

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FIGURE 5

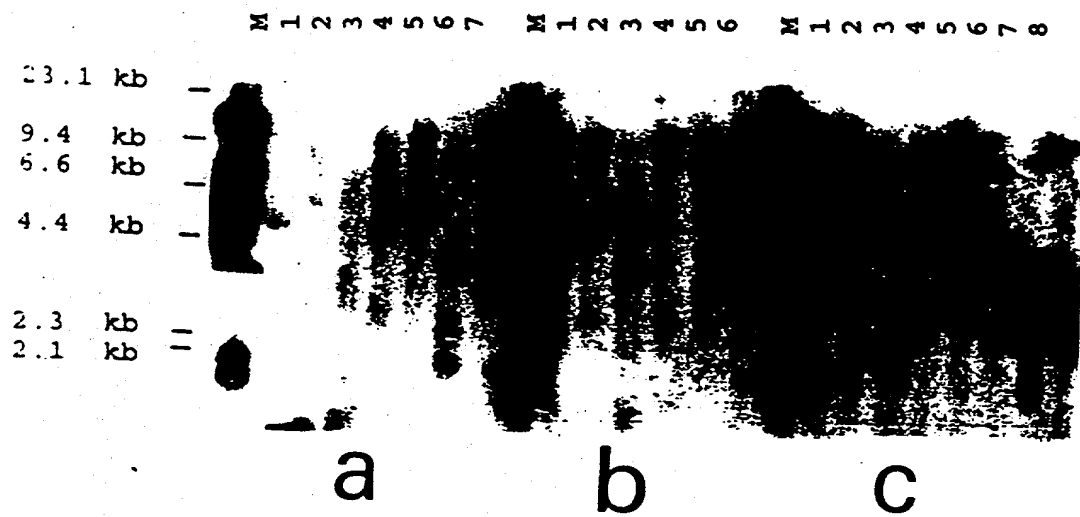


FIGURE 6



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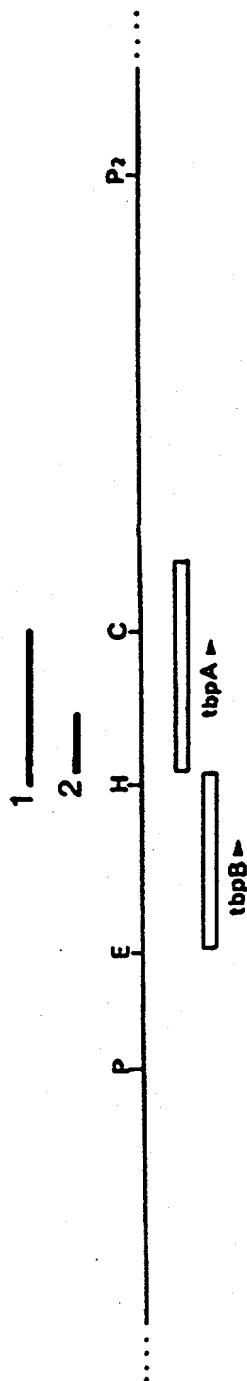
FIGURE 7



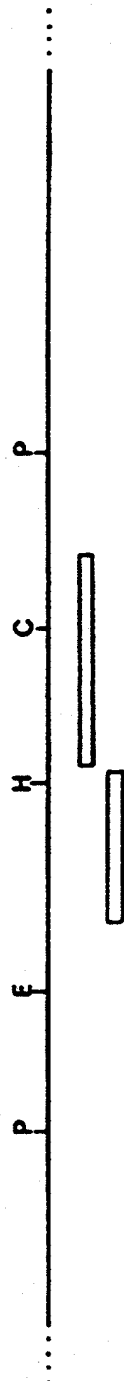
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FIGURE 8

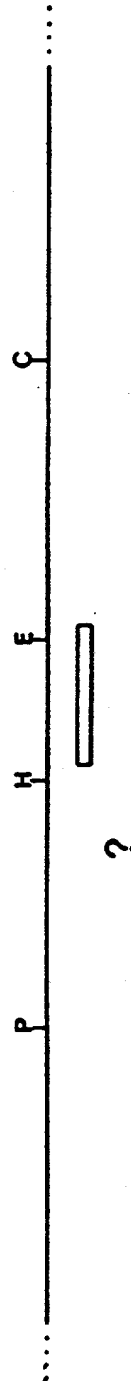
P. haemolytica A1



A. suis 3714



A. pleuropneumoniae CM5, Shope 4074



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[illegible]

PHTBP	MIMKYHHPFRYSTVALTVPALSHSYGAATENKKIIBENNDLAVLEDEVIVTE	50
NGTBP1	MQQQ-HLFRNLILCLSLMTALP-AYAENVQAGQAQEQKQ----	44
NM1	MQQQ-HLFRNLILCLSLMTALP-VYAENVQAEQAQEQKQ----	44
	* . . . * . . . * . . . * . . . * . . . * . . . *	
PHTBP	SHYAHERQNEVTGLGKVVKNYHEMSKNQILGIRDLTRYDPGISVVEQGRG	100
NGTBP1	KKQKTRRDNEVTGLGKLVKTADTLKSKEQVLDIRDLTRYDPGIAVVEQGRG	94
NM1	KKQKTRRDNEVTGLGKLVKSSDTLSKEQVLNIRDLTRYDPGIAVVEQGRG	94
 * * * * *	
PHTBP	ASSGYAIRGVDKNRVSLLDVGLPQAHSYHT--LSGD--ANGGAINBIE	144
NGTBP1	ASSGYSIRGMDKNRVSLTVDGLAQIQSYTAQAALGGTRTAGSSGAINBIE	144
NM1	ASSGYSIRGMDKNRVSLTVDGVSQIQSYTAQAALGGTRTAGSSGAINBIE	144
	***** * * * *	
PHTBP	YENIRSIELSKGASSABYGGSGAHGGAIGFRTKDAQDIIKEGQHWGLDSKT	194
NGTBP1	YENVKAVEISKGSNSVEQSGALAGSVAFQTKTADDVIGEGROWGIQSKT	194
NM1	YENVKAVEISKGSNSSEYGNALAGSVAFQTKTAADIIGEGKQWGIQSKT	194
	***** * * * *	
PHTBP	SYASKNSHFLQ-IAAAGEAGGFALVIATHRHGKETKIHSEANKLKHNIR	243
NGTBP1	AYSGKNRGLTQSIALAGRIGGAEALLIRTGRHAGEIRAHEAAGRGVQSFN	244
NM1	AYSGKDHALTQSLALAGRSGGAEALLIYTKRRGREIHAKHDAGKGVQSFN	244
	***** * * * *	
PHTBP	RITGFENRYDFTQIPHRMPPGGS---FFIVEDTCPTLDCTPRARVKLNRD	290
NGTBP1	RLAPVDD-----GSKYAYFIVEEBECKNGGHE-KCKANPKKD	279
NM1	RLVLDEDKKE-----GGSQYRYFIVEEBECHNG-YA-ACKNKLKED	282
	* * * * *	
PHTBP	NFPVRTFPBYTPEERNABQIPYRTEQLSAQEKTKGDRIAPNPLDYKSNSV	340
NGTBP1	VV-----GEDKRQT-----VSTRDYTGPNRFLADPLSYBSRSW	312
NM1	AS-----VKDERKT-----VSTQDYTGSNRLLANPLEYGSQSW	315
 * * * *	
PHTBP	FMKPGYHFNS-SHYLGAILEDTKQR-TISVICKRQLTIQKTLTYHLGTM	388
NGTBP1	LFRPGFRFENKRHYIGGILERTQQTFTDRDMTPVPAFLTAKAVFDANQKQAG	362
NM1	LFRPGWHLDN-RHYVGAVLERTQQTFTDRDMTPVPAYFTSSEDYVP-----G	359
	... * * * * *	
PHTBP	FMKGIIFRWLSVQAK-DPLWVAHMPCEV-----DERHHKRRLGF	426
NGTBP1	SLRGNGNHKYAGNHKYGGFLTSGENNA PVGA EYGTGVFYDETHTKSRYGL	412
NM1	SLKGLG--KYSGDNKAERL FVQGE GSTLQIGYGTGVFYDERHTKNRYGV	407
	.. * * * * *	
PHTBP	TYKY-KPENNRWLDINS CVRALRSRCCALSKQDIELYSRLHRLHCS DYP	475
NGTBP1	EYVYTNA DKDTWAD----YARL-----SYDRQIGLDNHPQQTHCSA-D	451
NM1	EYVYHNA DKDTWAD----YARL-----SYDRQIGLDNRLQQTHCSH-D	446
	* * * * *	
PHTBP	VVDKNCGP TLDKSWSMYRTERNNYQEKHRVHLEFDLALNAGQGVFLQTH	525
NGTBP1	GSDKYCRPSADKPF SYKSDRVIYGESHKLLQAAPKKSFD TAK----IRH	497
NM1	GSDKNCRPDGNKPYSFYKSDRMIYBESRNLFQAVFKAFDTAK----IRH	492
	***** * * *	
PHTBP	KLNLGLGFBSINSLMDHGDMTAQYTLGRLYQL---PRRDPRSIWTVSLCN	572
NGTBP1	NLSVN LGYDRFGSNLRHQDYQQSAN-RAYSLKTPPQNGKKTSPNGREK	546
NM1	NLSINLGYDRFKSQLSHSDYYLQNAV-QAYDLITPK----KPPFPNGSKD	537
	***** * * *	

FIGURE 9 CONT'D

PHTBP	NT-----RATLNCDAL-NLGIRLYLRCCLINQLNNPRYGSVLPQFGTRV	615
NGTBP1	NPYWVSI GRGNVVTRQICLFGNNTYTDCTPRS INGKSYAAV--RDNVRL	594
NM1	NPYRVSIGKTTVNTSPICRFGNNTYTDCTPRNIGGNGYAAV--QDNVRL	585
	*. *	
PHTBP	HRTWTP TSLGELPSIRAMAHYVNHHPNQVFWGRGAVKHLT-----LLSS	659
NGTBP1	GR-WADV GAG----LRY--DYRSTHSDDGSVSTGTHRTL SWNAGIVLKPA	637
NM1	GR-WADV GAG----IRY--DYRSTHSEDKSVSTGTHRNLSWNAGVVLKPF	628
	* * . . . * . * . . . * . * . . . *	
PHTBP	PWM-LKFAASG--RHVTL SVISG-ATDRFLVPPLILTGVNYKNES---YV	702
NGTBP1	DWLDLT YRTSTGFRLPSFAEMYGWRSGDKIKAVKIDPEKSFNKEAGIVFK	687
NM1	TWMDLT YRASTGFRLPSFAEMYGW RAGESLKTLDLKEKSFNREAGIVFK	678
	*. * . . * . * . . . * *	
PHTBP	SAIYNVDVRYCKTLY-----YRGQQLGDRATGQAKPDGY---QLHRFA	742
NGTBP1	GDFGNLEASWFNNAYRDLIVRGYEAQIKDGKEQVKGNP-AYLNAQSARIT	736
NM1	GDFGNLEASYFNNAYRDLIAPGYETRTQNGQTSASGDP-GYRNAQNARIA	727
	. . . * * * . * . *	
PHTBP	APG-----RNFSYHSKKFRPAK---ENTKNAESIFS	770
NGTBP1	GINILGKIDWNGVWDKLPEGWYSTFAYNRVRVRDIKKRADRTDIQSHLFD	786
NM1	GINILGKIDWHGVWGGLPDGLYSTLAYNR IKVKDADIRADRTFVTSYLF	777
 * * *	
PHTBP	A-----FFVGSNGLHTNSKSCFNGLRHEPIPYFFNFLRNVPFRFNEYHCCC	815
NGTBP1	AIQPSRYVVGSGYDQPEGKVGVMGLT-----YSKAKEITELLGSR---	827
NM1	AVQPSRYVLGLGYDHPDGIWGINTMFT-----YSKAKSVDELLGSQ---	818
	* . . * * *	
PHTBP	TSLIAASILHHIYHWVDFRYYYFV--YFCWILHHLIHINSFLMLLSHY	863
NGTBP1	-ALLNGNSRNTKATARRTRPWYIVDVSGYYTVKKHFTLRAGVYNLLNHRY	876
NM1	-ALLNGNANAKKAASRRTRPWYIVDVSGYYNIKKHFTLRAGVYNLLNYRY	867
	* * * *	
PHTBP	--REVVYLTCACAFNI VTVNGF----CVGCCSNILAEMKF	898
NGTBP1	VTWENV RQTAAGAVNQHKNVGVYNRYAAPGRNYTFSLEMKF	917
NM1	VTWENV RQTAGGAVNQHKNVGVYNRYAAPGRNYTFSLEMKF	908
	* * * * *	

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FIGURE 10

PHTBP1	MIMKYHFRYSTVALTVLFAISHSYGAATENKKIERNNDLAVLDEVIVTE	50
APL	MHPKLNPY---ALAFSLFLVACSGGKGSFD-----LEDVRPNQ	36
APL7	MHPKLNPY---ALAFSLFLVACSGGKGSFD-----LEDVRPNK	36
	* * *	
PHTBP1	SHYAHERQNEVTGLGKVVKNYHEMSK--NQILG--IRDTRYDPGISVVE	96
APL	TAKAEKATTSYQDEBTKKTKBBLDKLMEPALGYETQILRRNKAPKTETG	86
APL7	TGVSKER--YKOVETAKKEKEQLGELMEPALGYVVKV-----PVSSFE	78
 * *	
PHTBP1	QGRGASSGYAIRGVDKNRVSLLDGLPOAHSYHTLSGDANGGAINBI-BY	145
APL	EKRNER----VVELSEDKITKLYQESVEIIPH--LDLNGKTTSDNDVYHS	130
APL7	NKK-----VDISD-----IEVITNGNLDDVPYKANSSK-YNV	109
 * *	
PHTBP1	ENIRSIELSKGASSAEYGGGAHGAIGFRTKDAQ-DIIEG-----	185
APL	HDSKRLDKNRDLKYVRSGYVYDGSFNEIRNDSGFHVFKQIDGYVYYLG	180
APL7	PDIK--TKDSSLQYVRSGYVIDGKHSGSNEK-----GYVYYKG	145
 * *	
PHTBP1	----QHWGLDSKTSYASKNSHFLQIAAAGEAGGFALVIATHRH-----	225
APL	VTPSKELPKGKVISYKGTWDFVSNINLREIDGFDTSGDGNVSATSITE	230
APL7	NSPAKELPVNQLLTYTGSWDFTSNANLNNE-----BGRPNYLNDDYYTK	189
 * *	
PHTBP1	--GKETKI-HSEANKLKHNIIRITGFENRYDFTQIPHRMPGGSPFFIVED	272
APL	TVNRDHKVGEKLGDNVKGVAHSSEPAVDPNKILTGSLYRNG--YINRN	278
APL7	FIGK--RVGLVSGDAKPAKHKYSQFEVDFATKMTGK-----SD	228
 * *	
PHTBP1	TCPTLDCTPRARVKLNDRNFFVVRTFPEYTPERNABEQIPYRTEQ--LSAQ	320
APL	KAQEVTKRYSIEADIAGNRFRG-----KAKAEKAGDPIFT-DSNYLEGG	321
APL7	KEKTI---YTVNADIRGNRFTGAATASDKNKGKESYNFFSADSQSLEGG	275
 * *	
PHTBP1	EKTGKDRIAPNPLDYKNSVFMKFGYHFNSSHYLGAILEDTKQRTISVIC	370
APL	FYGPKEBEMAGKFTNNKSLFAVFAAKSENGETTERIIDATKIDLTQFN	371
APL7	FYGPKEBEMAGKFVANDKSLFAVPSAKENGSNVNTVRIIDASKIDLTNFS	325
 * *	
PHTBP1	KRQLTI--QKTIITYHLGTMFMKGIIIFRLSVQAKDPLMVAHMPCEVDER	418
APL	AKELNPNFGDASVLIIDGQKIDLAGVNFNSKTVEINGKTMVAVACCSNLE	421
APL7	ISELANFGDASVLIIDGKKIKLAGSGFTNKHTIEINGKTMVAVACCSNLE	375
 * *	
PHTBP1	HHKRRLGFTYKYKPNNRWLDINSVCVRALRSRCCALSQODIELY-----	463
APL	YMK----FGQLWQKEGKQOVKDNSLFLQGERTATDKMPAGGNYKYVGTWD	467
APL7	YMK----FGQLWQAEGBGKPNNSLFLQGERTATDKMPKGGNYKYIGTWD	421
 * *	
PHTBP1	---SRLHRLHCSDPVVDKNCGPTLDKSWSMYRTERNNYQEKATCHSFCI	510
APL	ALVSKGTNWIAEADNNRESGYRTEFDVNFSDKKVNGKLPDKGGVNPVFTV	517
APL7	AQVSKENNWVATADDDRKAGYRTEFDVDFGNKLSGKLPDKNGVNPVFTV	471
 * *	
PHTBP1	LKALNAGQGVFKQTHKLNGLGFESNLIRLTIIGIILPNIPKAGYTSYRG	560
APL	DATIN-GNGFIGSAKTSDSGFALDAGSSQHGNAVPSDIKVNGGFGYGP--T	564
APL7	DAKID-GNGFTGKAKTSDEGFALDSGSSRYENVKPNDAVAVSGFGYGP--T	518
 * *	
PHTBP1	RGRLDNPIYIRDRPSIETVSLCNTRATLLLLRVNKGIRLLLR	604
APL	AGELGGQFHHKSDNGSVGAV-----FGAKRQIE---K	593
APL7	AABLGGQFHHKSENGSVGAV-----FGAKQOVK---K	547
 * *	

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FIGURE 11

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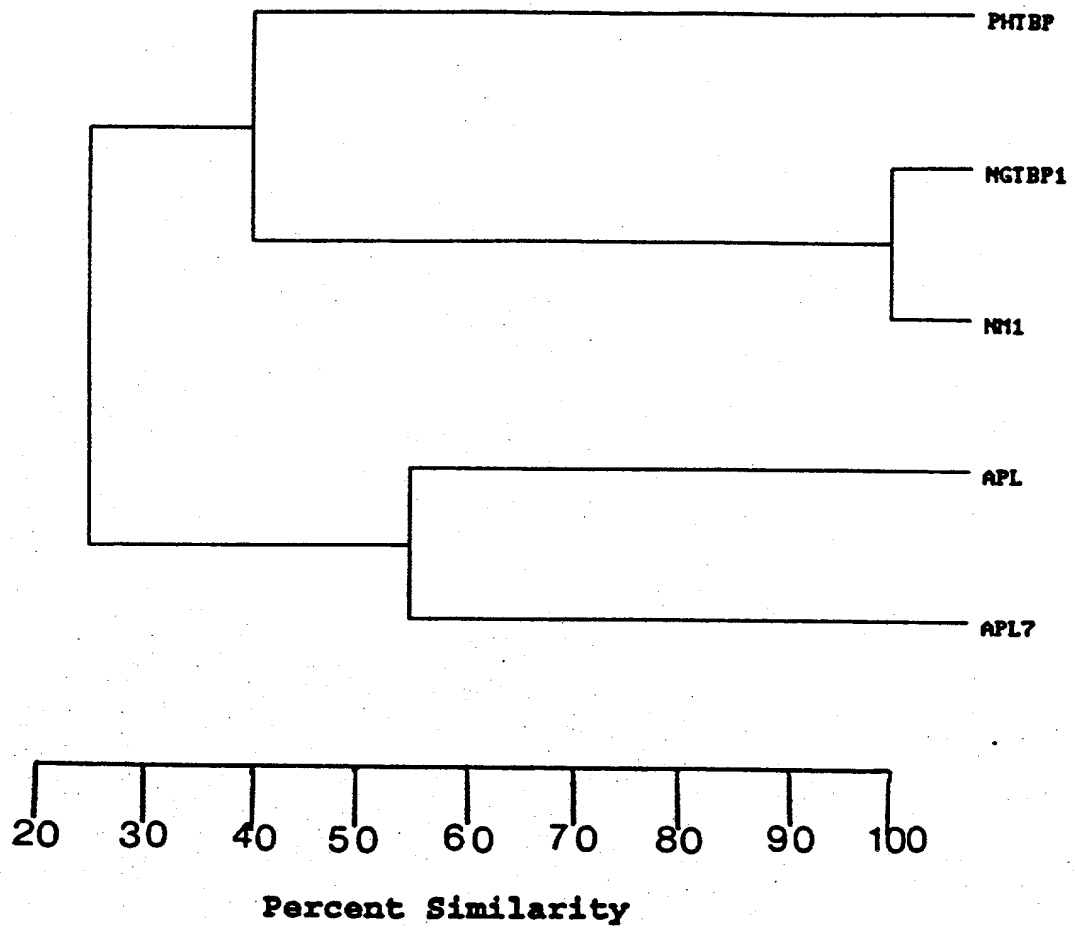
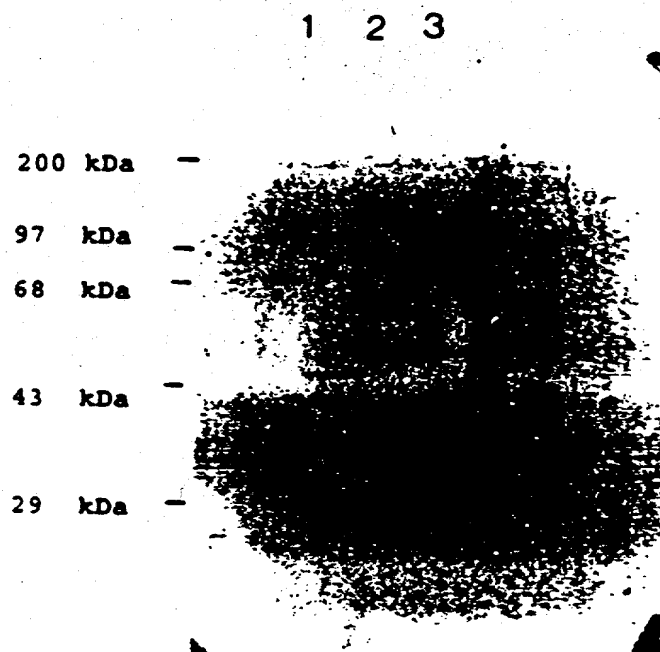


FIGURE 12

1		TonB box	
PHTBP1	DEVIVTE	22	
CIRP	ETMVVTA	12	
FEPAP	DTIVVTA	17	
FHUAP	DTITVTA	13	
FECAP	FTLSVDA	30	
	. . *		
2			
		YAIRGVD---KNRVSLLDVG	121
		VSIRGLDSS---YTLILVDG	105
		IDIRGMGPE---NTLILIDG	102
		LIIRGFAAEGSQNNYL-NG	141
		FGIRGLNPRLTSRSTVMDG	199
		*** . * *	
3			
		IELSKGASSAERYGSGAHGGAIGFRTKD	177
		IEVVRGPMSSLYGSDALGGVVNIITKK	159
		IEVLRGPARARYGNGAAGGVVNIITKK	180
		AEIMRGFVSVLYGKSSPGGLLMVSKR	188
		IDVVRGGGAVRYGPQSVGGVVNFVTRA	251
		. . . * * *	
4			
		FKQTHKLNGLGLF	533
		PETSESWELGLYY	461
		AETSINKKEIGLEF	447
		PSKGKQYEVGVKY	560
		PEKARTWELGTRY	578
	 *	

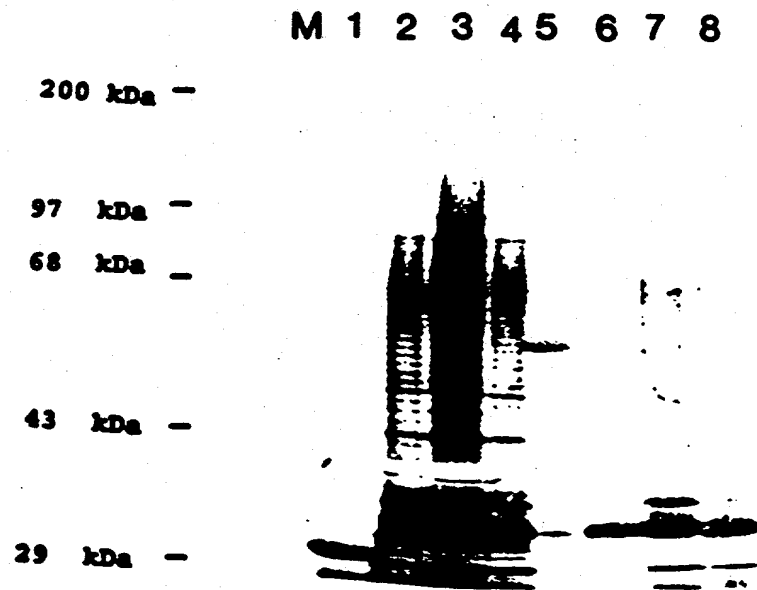
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FIGURE 13



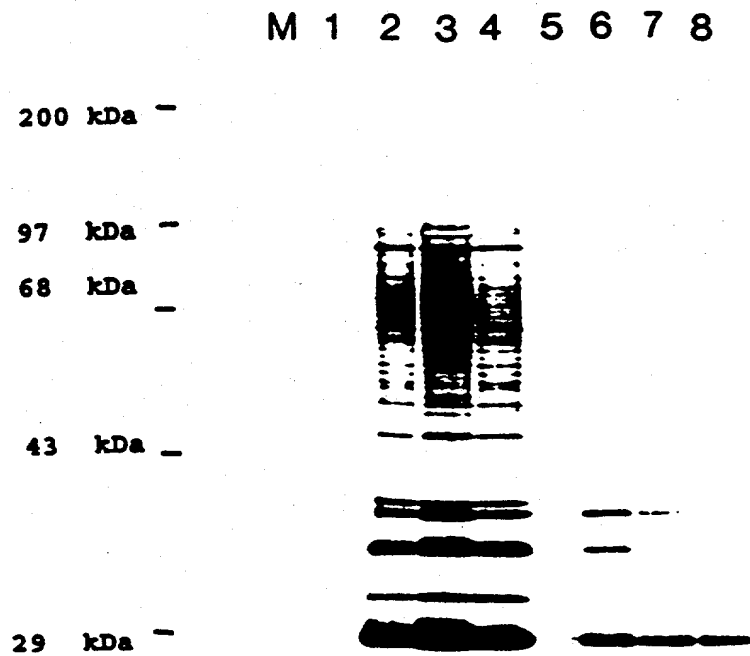
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FIGURE 14



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FIGURE 15



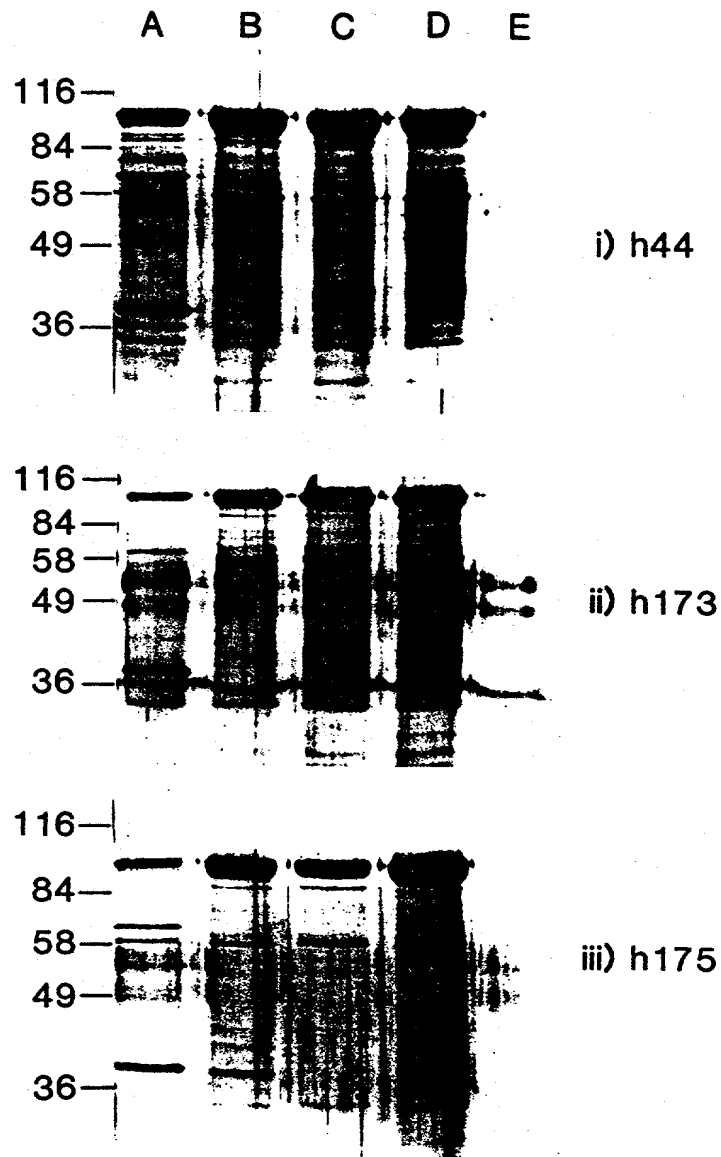
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FIGURE 16

	HRP- bTf	HRP- oTf	HRP- cTf	HRP- eTf
h173 -	●	●	●	
h174 -	●	●	●	
h175 -	⊙	●	●	
h176 -	●	●	●	
h44 -	●	●	●	
h50 -				●

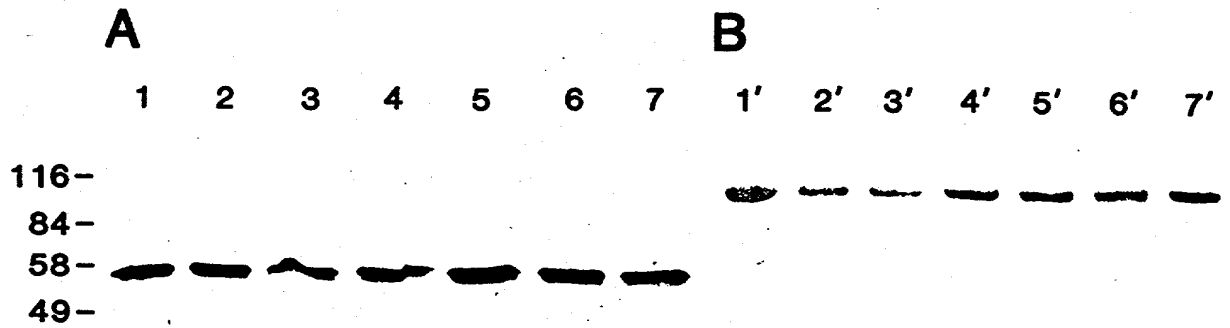
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FIGURE 17



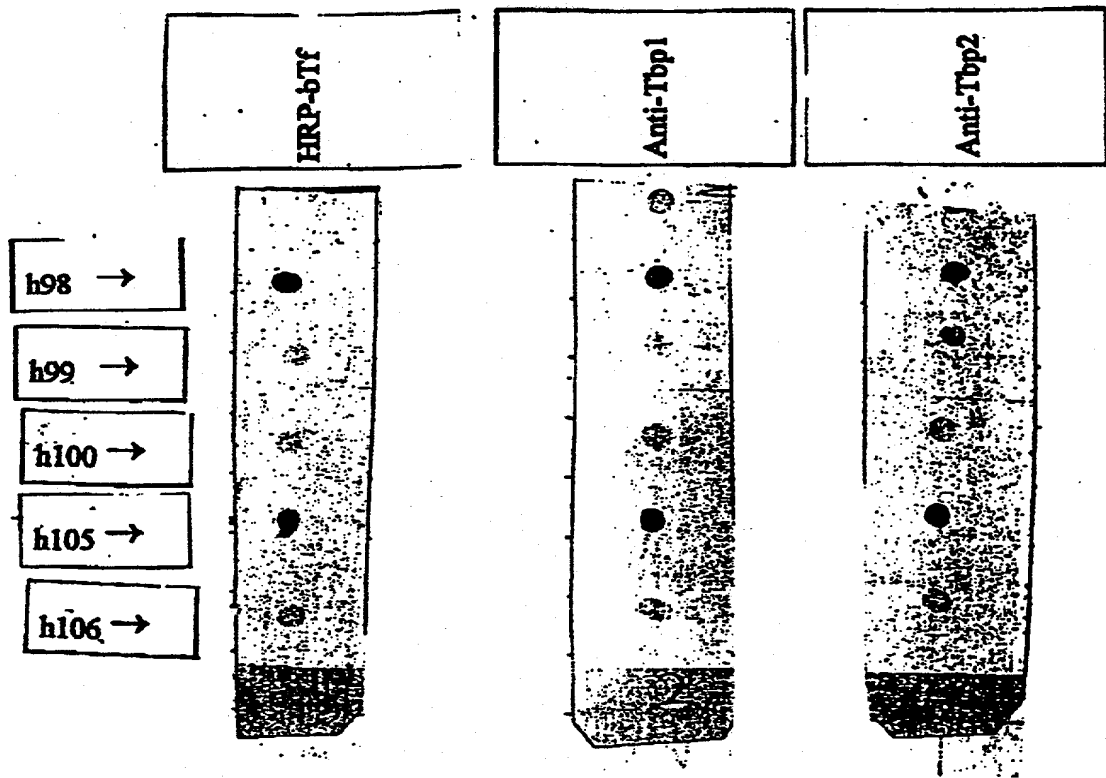
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FIGURE 18



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FIGURE 19



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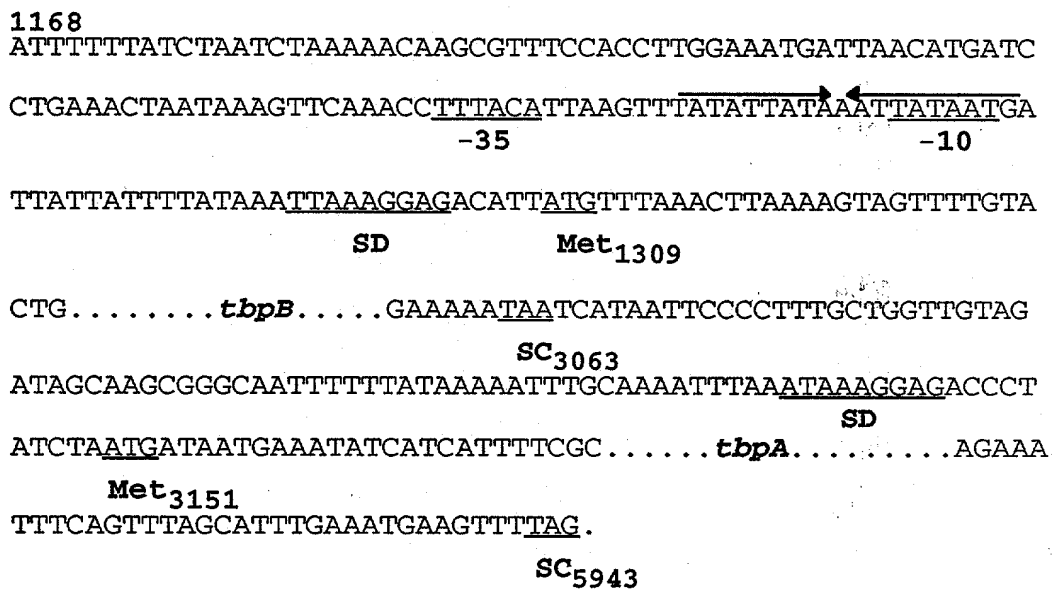
[illegible]

FIGURE 21

ATGATAATGAAATATCATCATTTTCGCTATTACCTGTTGCCTTAACAGTGTTATTTGCTC
TTTCTCATTCATACGGTGCTGCGACTGAAAATAAAAAAATCGAAGAAAATAACGATCTAGC
TGTTCTGGATGAAGTTATTGTGACAGAGAGCCATTATGCTCACGAACGTCAAAACGAAGTA
ACTGGCTTGGGGAAAGTAGTGAAAATTATCACGAAATGAGTAAAAATCAAATTCCTGGTA
TTCGTGATTTAACTCGCTATGACCCTGGTATTTTCGGTGGTGGAACAAGGTCGCGGTGCAAG
TAGTGGCTATGCCATTCGAGGTGTAGATAAAAACCGTGTGAGCTTACTTGTGATGGGCTA
CCACAAGCGCACAGTTATCATACGCTAGGTTTCAGATGCTAATGGTGGTGCAATTAATGAGA
TTGAGTATGAAAACATTCGTTCAATTGAGTTAAGCAAAGGAGCAAGTTCTGCGGAATATGG
CTCTGGTGCGCATGGTGGTGCTATTGGTTCGTTTTCGTACTAAAGATGCGCAGGATATTATTAAA
GAGGGGCAGCATTGGGGCTTAGATAGTAAGACCTCTTATGCCAGCAAAAATAGCCATTTT
TACAGTCTATCGCAGCGGCTGGTGAGGCGGGTGGTTCGTAAGCACTTGTTATTGCAACTCA
CCGACACGGTAAAGAGACCAAAATTCATTCCGAGGCAATAAATTAAAACATAATATTCGG
CGTATAACCGGCTTTGAAAATCGCTACGACTTTACCCAAATTCGCGACAGAATGCTCCTGG
AGGATCTCCTTTTAATTGTGGAAGATACTTGCCCAACATTAGATTGTACTCCTCGTGCAAG
GGTTAAGTTGAACCGCGATAATTTCCAGTGAGAACATTTCCGGAATATACGCCTGAAGAG
CGCAAACAGCTTGAGCAGATTCTTATCGCACTGAGCAGCTCTCAGCCCAAGAATATACCG
GTAAAGATCGCATTGCACCAACCCCTTAGATTACAAGAGTAATTCGTTTTTATGAAGTT
TGGCTATCACTTCAACTCGTCTCATTATCTTGGCGCAATCTTAGAGATACAAAAACACGC
TACGATATCCGTGATATGCAACGCCAGCTTACTATACAAAAGACGATATTAACTTATCAC
TTAGGAACATATGTTTATGAAGGGGATAATATTTTAGATGGCTTAGTGTTCAAGCCAAGGAT
CCCTTATGGGTGCGCTATAGCCATGTGAAGTTTTTTGATGAACGTCACCACAAACGTCGT
TTAGGATTCACCTATAAATATAAACCAGAGAATAATCGCTGGTTGGATAGCATTAAACTCA
GTGCGGATAAACAAGATATTGAACATATAGCCGGCTACATCGCTTGCATTGTAGCGATTA
TCCTGTGGTAGATAAAAATTGCCGCCCGACTTTGGATAAATCTTGGTCTATGTATCGAACT
GAGCGTAATAATTACCAAGAAAAGCATCGTGTCAATTCATTTAGAATTTGATAAAGCGCTAA
ATGCTGGTCAAGGCGTATTTAACCAAAACCACAACTGAATTTAGGGTTGGGCTTTGATCG
ATTTAATTCGCTTATGGATCATGGGGATATGACTGCCCAATATACCAAAGGCGGTTATACC
AGCTACCGCGGTAGAGGGCGTTTAGATAATCCATATATTTATCGCCGCGATCCACGCAGTA
TTGAAACGGTATCTTTGTGTAATAATACACGCGGCGACATCTTAACTGTGAACCGCGTAA
AATTAAAGGCGATAGCCATTTTGTAGCTTCCGCGATCTAGTGATAAGCGAGTATGTGGAT
TTGGGATTAGGGGTGCGTTTTGATCAACATCGATTTAAATCTGATGATCCGTGGACACTTA
GCCGAACCTTATCGAAATTGGTCTTGGAATGGTGGGATTACGCTTAAACCAACAGAGTTTGT
ATCGCTTTCTTATCGCATTTCAAACGGTTTTAGAGTGCTGCATTCTATGAACCTTATGGT
AAACGTGATCATATTGGGCTTAAAGATAACGAATATGTGCAACGCGCGCAACGTAGCCACC
AGTTAGAGCCAGAAAAATCGACTAATCATGAGATTGGAGTTAGCTTTAAAGGTCAATTTGG
TTACCTTGATGTGAGCTATTTCCGTAATAACTATAAAAATATGATTGCGACAGCATGTAAA
AGAATAATACAAAAATCACACTGTTTCTATAACTACCATAATATTCAAGATGTAGCACTAA
ACGGGATAAATTTAGTCGCTAAATTTGACTTACACGGTATTTTATCTATGCTGCCAGATGG
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AGACTCGATAGCGTAAACGATCCTATTCTAGATGCGATTTCAGCCAGCACGCTATGTGCTTG
GATTCGGCTACGATCACCCAGAAGAAAAATGGGGAATTGGCATTACTACCACCTATTCTAA
AGCCAAAAACGCCGATGAGGTGGCAGGCACACGTCATCACGGNATACATCGCGTTGATTTA
GGTGGCAAACTGACCGGTTCTTGGTACACCCATGATATTACCGGTTACATCAATTATAAAA
ACTACACCTTACGTGGAGGAATTTATAATGTGACTAATCGTAAATATTCCACTTGGGAATC
AGTGCGCCAATCCGGTGTGAATGCAGTAAACCAAGACCGGGTAGCAATTACACTCGATTT
GGCGCTCCGGGGAGAAATTTCAGTTTAGCATTTGAAATGAAGTTTTAG

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FIGURE 22

1 MIMKYHHFRYSVALTVLFALSHSYGAA TE
 31 NKKIEENNDLAVLDEVIVTESHYAHERONE
 61 VTGLGKVVKNYHEMSKNQILGIRDLTRYDP
 91 GISVYEQGRGASSGYAIRGVDKNRVSLLD
 121 GLPQAHSYHTLGSDANGGAINIEIENIRS
 151 IELSKGASSAEYGS GAHGG AIGFRTKDAQD
 181 I I KEGQH WGLDSKTSYASKNSHFLQSI AAA
 211 GEAGGF EALVIATHRHGKETKI HSEANKLK
 241 HNI RRITGFENRYDFTQIPHRMLLEDLLI
 271 VEDT CPTLDCTPRARVKLN RDNFPVRTFPE
 301 YTP EERKQLEQIPYRTEQLSAQEYTGKDRI
 331 APNPLDYKSN S VFMKFGYHFNSS SHYLGAIL
 361 EDTKTRYDI RDMQTPAYYTKDDINLSLRNY
 391 VYEGDNILDGLVFKPRI PYGLRYS HVKFFD
 421 ERH HKRR LGFTYKYKPENNRWLD SIKLSAD
 451 KQDIELYSRLHRLHCS DYPVVDKNCRPTLD
 481 KSWSMYRTERNNYQE KH RVIHLEFDKALNA
 511 GQGVFNQTHKLN LG LGFD RENS LMDHGD MT
 541 AQYTKGGYTSYRGRGR LDNPYIYRRDPRS I
 571 ETVSL CNNTRGDILNCEPRK IKGDSHFVSF
 601 RDLVISEYVDLGLGVRF DQHRFKSDDPWTL
 631 SRTYRNW SWNGGITLKPTEFVSLSYRISNG
 661 FRVPAFYELYGKR DHIGLKDNEYVQRAQRS
 691 HQLEPEKST NHEIGVSFKGQFGYLDVSYER
 721 NN YKNMIATACKRI IQKSHCFYNYHNIQDV
 751 ALNG INLVAKFDLHGILSMLPDG FYS SVAY
 781 NRVKYKERKLTDSRLDSVNDPILDAIQPAR
 811 YVLGFGYDHP EEWGIGITTTYS KAKNADE
 841 VAGTRHHGIHRVDLGGKLTGSWY THDITGY
 871 INYKNYT LRGGIYNVTNRKYSTWESVRSQSG
 901 VNAV NQDRGSNYTRFGAPGR NFSLA FEMKF

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FIGURE 23

ATGTTTAAACTTAAAAGTAGTTTTGTACTGCTTAATGCGGCGCTACTTGCTGCTTGTTCCT
CAAATGGTGGAAGCTTTGATGTTCAATCTGCCAAAGTTGAATCTCAAACGCAAACCTACCCC
CAAAAAGCCAAGTTTACAAGATGATAATAGTAACGCAAGACGTACAGTAAGCGCTTCTGAA
ACTGAAGCTTTATTGCAGCCGGGGTTTGGTTTTTTCAGCCAAAATTCGCGTCGTAATCTCC
TTCCGCAGGGGAAGGAAGATGTAGCCCCATTGGTGATATAAAAGAGATTACTGGAGATCT
GCCAAAAATTCGTATGAAGAAGAGGTTAAAGCGTGCGGTAGTAGTGTGATGGATTTAGC
CATACTCATGATAGAAATCATAAGTTGTATACAAGAGATTTTAATTTTGTTCGTTCCGGCT
ATGTTGTGCATTCTGGTCCAAAACCTGAAATAAAGCCTAAAGAAATTTTGAGAACAGGTGC
ACATGGGTATGTTTACTATTTAGGTATAGAGCCGCCCAAAGCAATACCTACCCAAAAACTA
ACTTATAAAGGATATTGGGATTTTACTACCTATGCGGCTAAGGGGAGAGATAGTAATATTT
TTCTAATTCCTCGCAGGCATCAATAGTGGCGCCATACCGGAAAATAGTCACGATATTAATGT
TGATGATTCTGAAAAACCAATGGGGCATAACAGGAGAAATTTACGGCTGATTTTGTCTAATAAA
ACTTTAACTGGAACATTGGTTTCGTAATGGGTATGTTAGTCGTAGCAAAGAGCAAAAAATTA
CAACAATTTACGATATTGATGCGAAAATTAAGGTAATCGCTTTTCTGGTAAAGCAAACCC
AAAAAAACCGATGATCCTTATTTTTGGGAAAAGCTCCACGACACTTGAAGGTGGATTTTTT
GGTGGGGAGGCTCAAGAACTTGCCGGTAAATTCCTAGCTGATGATAAGTCGGTATTTGTG
TTTTTGCTGGCACACGAGATGCTAAAAAAGATGATAGTGAATCTGCCTTTGATGCTTTCCC
AATTAACTTAAAGATTTAAATAAATCTGAGATGGATACTTTTCGGGAATGCGACACATTTG
ATTATTAAACAATAAGCAGATTCCACTTATTGCGGAAGCCACAAAAGCTTTGCCGAGATGA
AATTTGATGATTTGGTTACCCGTAATTTGATGGAAAAACGTATCGAGTTTCAGTCTGCTG
TAATAATTTAGATTATGTCAAATTTGGGATTTATAGCGAGGGAAATAATAGTGATACTGCT
CTCCAAGAATATTTAGTAGGAGAACGTACAGCTCTGGCAGATTTGCCAACAGGGACAGTAA
AATATCGAGGTACTTGGGACGGGGTAATGTACAGTAAATCTGGCTCGGCAGGGGTGAATC
GCCAAGTAACAGCGAAAGTGGTACTCGTTCACTATTTCGATGTAGATTTTGTCAATAAAAAA
ATTAATGGCAAGCTGATTGCTAATGATGGTGTGAAGAACGCCCAATGCTGACACTGGAAG
GCAATCTGAAAGGGAATGGTTTTGGAGGCACAGCCAAAACGGGCAATCTGGTTTTTAATCT
TGATCCCAAAAGTACGAATGGTGGCACGGTAGGGCATATAAATACTCAATTTGAAGGGGGC
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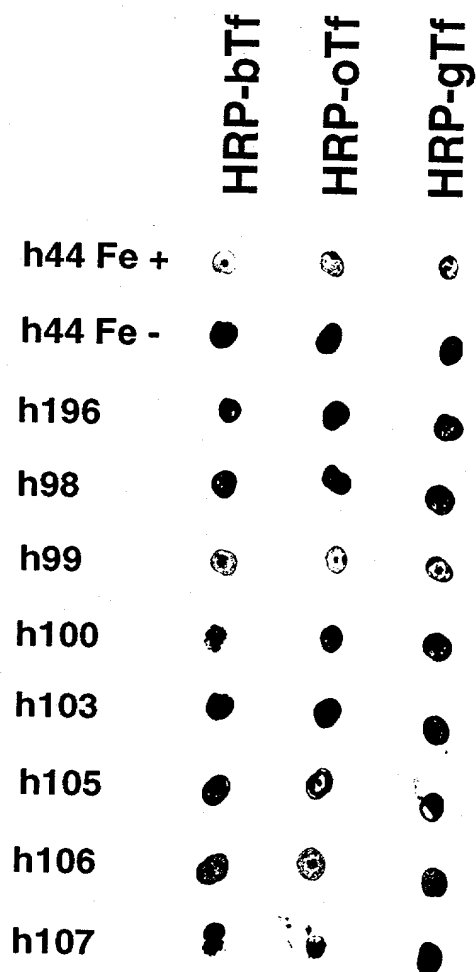
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FIGURE 24

1 MFKLKSSFLNAAALAACSSNGGSEDEVQS
 31 AKVESQTQTTPKKPSLQDDNSNARRTVSAS
 61 ETEALLQPGFGFS AKIPRRNLLPQGKEDVA
 91 PIGDIKEITGDLPKIPYEEEVKACGSSADG
 121 FSHTHDRNHKL YTRDFNFVRS GYVVHSGPK
 151 PEIKPKEILRTGAHGYVYYLGIEPPKAIP T
 181 QKLT YKGYWDF TTYAAKGRDSNIFLIPAGI
 211 NSGAIPENSHDINVDDSEKPMGHTGEFTAD
 241 FANKTLTGTLVRNGYVSRSKEQKITTIYDI
 271 DAKIKGNRFS GKANPKKPMILIFGKSS TTL
 301 EGGFFGGGEAQELAGKFLADDKSVFVYFAGT
 331 RDAKKDDSES AFDAFPIKCLKDLNKSEMDTF
 361 GNATHLIINN KQIPLIAEATKSFAEMKFDD
 391 LVTRTIDGKTYRVS VCCNNLDYVKFGIYSE
 421 GNNSDTALQEYLVGERTALADLPTGT VKYR
 451 GTWDGVMYSKSGSAGVESPSNSESGTRSLF
 481 DYDFVNKKINGKLIANDGVEERPMLTLEGN
 511 LKGNGEGGTAKTGNSGFNLDPKSTNGGTVG
 541 HINTQFE GGFYGPKATELGGIVQNTETDKD
 571 RV SITEGGKRQIEK

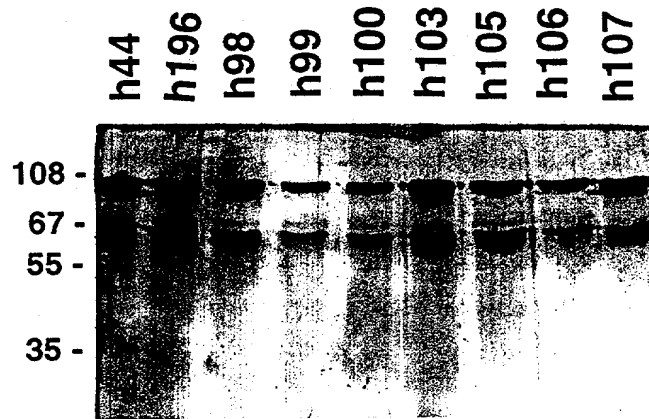
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FIGURE 25

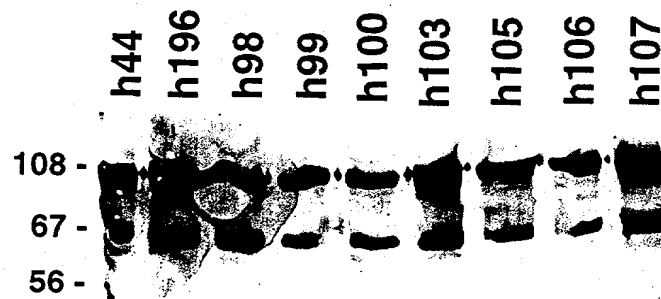


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FIGURE 26



A



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FIGURE 27

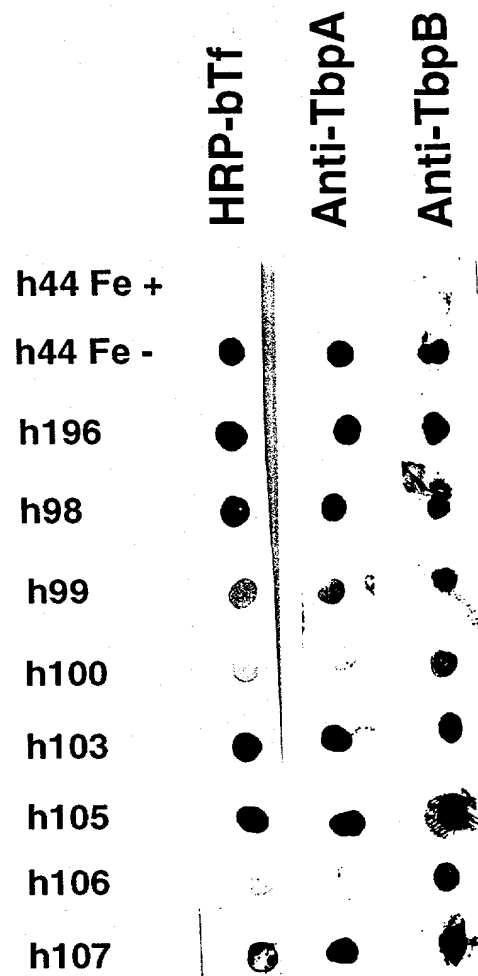
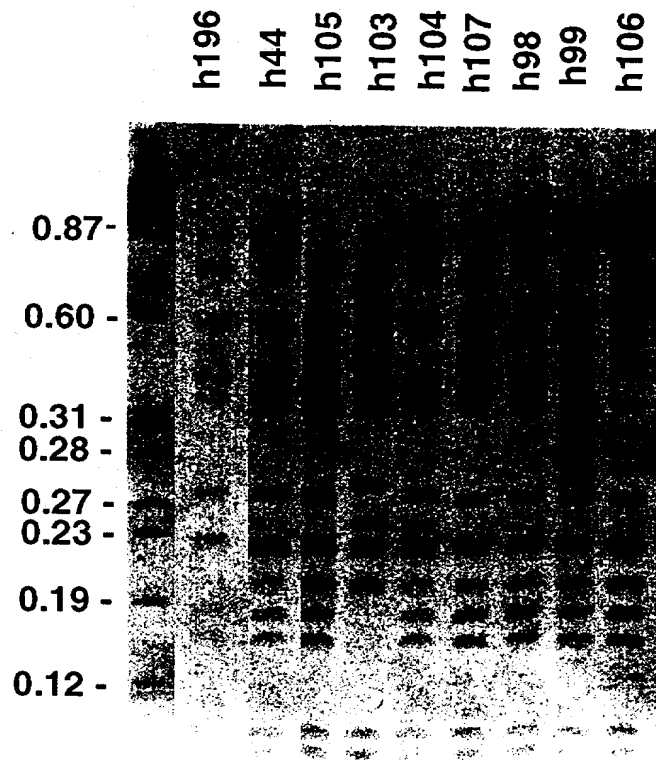
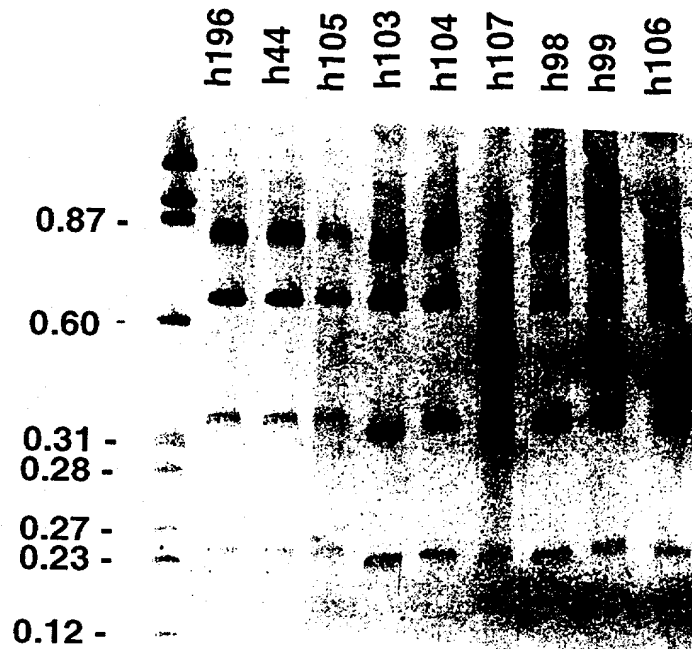


FIGURE 28



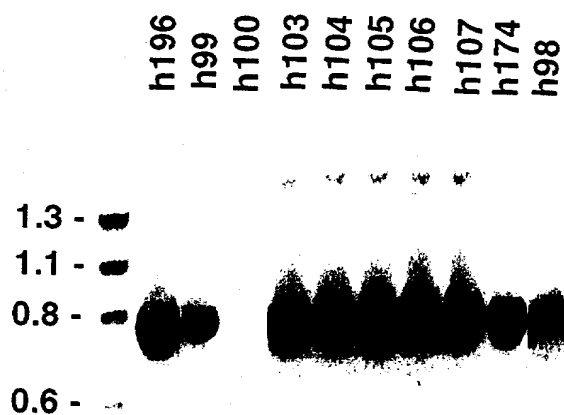
A



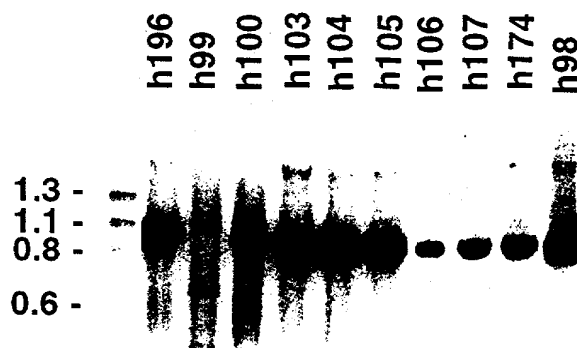
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FIGURE 29



A



B

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